Spatio-temporal control of cell division in fission yeast by Cdr2 medial cortical nodes

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SPATIO-TEMPORAL CONTROL OF CELL DIVISION IN FISSION YEAST BY Cdr2 MEDIAL CORTICAL NODES

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I would like to dedicate this thesis to five people:

Marabunta, Parabuntu, Moxi and Drupu.
(AKA Assumpta, Jordi, Motserrat i Andreu)

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David

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Hari Seldon knew, I was afraid of the Mule though.

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A. The cell cycle

The cell division cycle is the process that allows one cell to become two. It is used by all cellular live forms. It allows growth in cell number while conserving the genetic identity of the mother cell. We can divide the cell division cycle in two parts: mitosis and interphase. **Interphase** is devoted to growth and duplication of the genetic material. **Mitosis** includes the separation of the two copies of the genetic material in a process called karyokinesis. Mitosis is followed by cytokinesis which is the physical separation between the two daughter cells. The progression of the cell cycle is controlled by the **cell cycle machinery** and the fidelity of the whole process is ensured by **checkpoints mechanisms** that halt cell cycle progression when their requirements are not fulfilled.

1. Interphase: getting ready to divide

Before a cell divides, it must undergo a series of events that will ensure genetic integrity and cell viability in the next generation. The cell needs to grow enough so that after division the daughter cells are about the same size as the mother cell was at the beginning of her previous round of division. The cell also needs to duplicate its genomic material and make sure that there are no mistakes when copying the DNA, or correct them, in order to preserve the genomic integrity. In parallel to DNA replication, the main microtubule organizing center, the centrosome starts duplicating. Its duplication will be completed by the time the cells start cell division. The cell growth during the cell cycle is accompanied by the growth and replication of the cell organelles so that when the cells arrives at mitosis each daughter cell can also inherit a whole set of organelles. This is also a controlled process which follows the progress of the cell cycle (Chan and Marshall 2010; Imoto, Yoshida et al. 2011).

Interphase is thus classically divided in three phases which in a chronological order are: G1, S and G2. In G1 the newborn cell grows and synthetizes the necessary proteins and takes the decision of engaging into another cell cycle or not. If the cell decides to engage into a cell cycle, G1 phase will be followed by the S phase during which the genetic material will be duplicated. Finally in G2 phase the cell checks that the duplication went right and no mistakes were made, if necessary it will engage correction mechanisms.

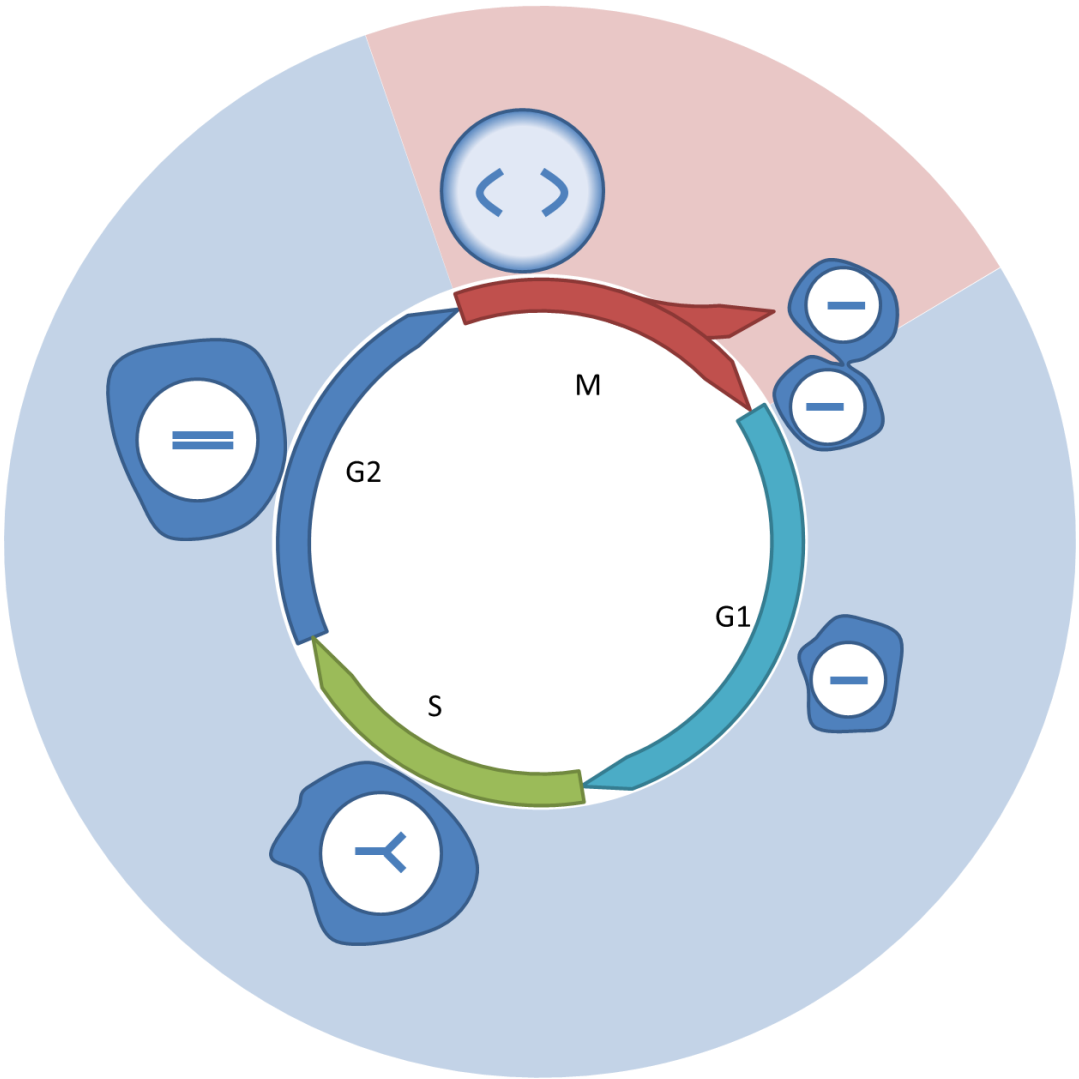


Figure1: Schematic representation of the eukaryotic cell cycle.
The blue part of the cycle represents Interphase with its three subdivisions: G1, S and G2. The red part represents Mitosis that ends with cytokinesis physically separating the daughter cells.

2. Mitosis: segregating cellular components in two equal sets

Once faithfully replicated, the genomic material needs to be segregated in two identical sets during mitosis. The separation of two copies of genetic material during mitosis is a multistep process mediated by the mitotic spindle. The spindle starts assembling in prometaphase while chromosomes condense. This is followed by nuclear envelope breakdown (NEB) in prometaphase, chromosome capture by the spindle during metaphase and segregation of sister chromatids, each containing one copy of the genetic material in anaphase.

Organisms that undergo a NEB have an open mitosis. In contrast yeasts like *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae* have a closed mitosis since their nuclear envelop remains impermeable and intact throughout mitosis. In this case, the mitotic spindle forms inside the nucleus. Intermediate situations can be found, such as in the case of *Schizosaccharomyces japonicus* which fenestrates its nuclear envelope and thus connect nucleoplasm and cytoplasm during mitosis without breaking the structure of the nuclear envelope (Yam, He et al. 2011).

The start of mitosis is triggered by the activation of the mitotic kinase Cdk1, whose activation will be described with detail later on, in this manuscript. The activation of Cdk1 promotes chromosome condensation and the separation of the duplicated centrosomes which will start forming the spindle between them. All this happens during **prophase**. The mitotic spindle is a microtubule-based, bipolar structure that provides the force and bidirectionality required for the separation of sister chromatids. The poles of the spindle are formed by centrosomes which nucleate most spindle microtubules. In yeasts the homologous structure are the Spindle Pole Bodies or SPBs. We will discuss these structures later on, in this manuscript. The complete formation of the spindle and the above mentioned rearrangements of the nuclear envelope happen during **Prometaphase**. During **Metaphase** the chromosomes are correctly attached by their centromeres which will build up protein platforms, the kinetochores. The role of the

kinetochores is to bind to the kinetochores microtubules of the spindle which will pull them apart. Until all the chromosomes are bi-oriented, unattached kinetochores produce a checkpoint signal that prevents the metaphase/anaphase transition. This control mechanism is the spindle assembly checkpoint (SAC) which assembles at the kinetochore. The SAC remains active until all kinetochores have been correctly attached and there is a uniform tension in each pair of sister chromatids. At this stage the cell has made the metaphase plate. Once the SAC turns off, another regulatory complex can switch on, the anaphase promoting complex (APC). The APC as its name states allows the cells to enter **Anaphase** by cleavage of the cohesin link that holds sister chromatid together. We will describe later on this manuscript how the APC works. The tension exerted by microtubules on kinetochores separate chromosomes in two movements: anaphase A, in which chromosomes are pulled towards the spindle poles by contraction of the kinetochore microtubules; and anaphase B, in which the spindles are further separated from each other by the elongation of interpolar microtubules. The chromosomes then start decondensing and the nuclear envelope reassembles. This last phase corresponds to **Telophase**.

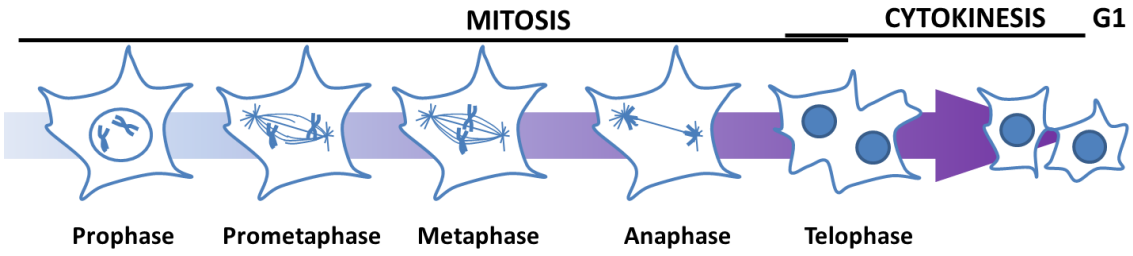


Figure2: Mitotic phases. (Adapted from (Rhind and Russell 2012))
Prophase: Chromosomes condense, and the centrosomes begin to separate to initiate spindle assembly.
Prometaphase: the nuclear envelope breaks down, the mature spindle is formed and spindle microtubules start capturing kinetochores.
Metaphase: When both kinetochores on a pair of sister chromatids are attached to microtubules from opposite spindle poles, opposing forces pull the pair to the metaphase plate at the middle of the spindle in a "bi-oriented" configuration. Until all the chromosomes are bi-oriented, unattached kinetochores produce a checkpoint signal that prevents the metaphase/anaphase transition.
Anaphase: the chromosomes separate until they reach the spindle poles.
Telophase: the chromosomes decondense and the nuclear envelope reassembles.

3. Cytokinesis: making two independent cellular entities

Cytokinesis is the final step of cell division which physically and irreversibly separates the mother cell in two daughter cells. Since it is irreversible, cytokinesis has to occur at the proper time and in a properly defined position and orientation in order to avoid chromosome segregation defects. The division plane is always positioned perpendicular to the mitotic spindle and cytokinesis always happens after nuclear division is accomplished.

Cytokinesis happens differently in different kingdoms. In plants the division is accomplished by formation of a phragmoplast, which is a microtubule based structure that organizes the secretion of materials required for the formation of the new cell wall that will separate daughter cells. Noteworthy, of all the living organisms, plants are the only organisms where cytokinesis does not depend on a contractile apparatus.

In contrast, contractile apparatus of different kinds have been described in bacteria, metazoans, fungi and archaea. In rod-shaped bacteria like *Escherichia coli* or *Bacillus subtilis* a ring made of the tubulin-like protein FtsZ is in charge of the cytokinetic process. Since molecular motors of the kinesin and dynein families have not been identified in bacteria it is thought that the contractile force is generated by FtsZ dynamics. Conformational changes in the FtsZ protofilaments may increase the curvature of the ring, which leads to a smaller diameter of the furrow. In metazoans and fungi the contractile apparatus is an F-actin-based ring that is attached to the plasma membrane of the cell. Myosin motors are thought to provide the necessary force for the constriction of the ring (Almonacid and Paoletti 2010; Balasubramanian, Srinivasan et al. 2012).

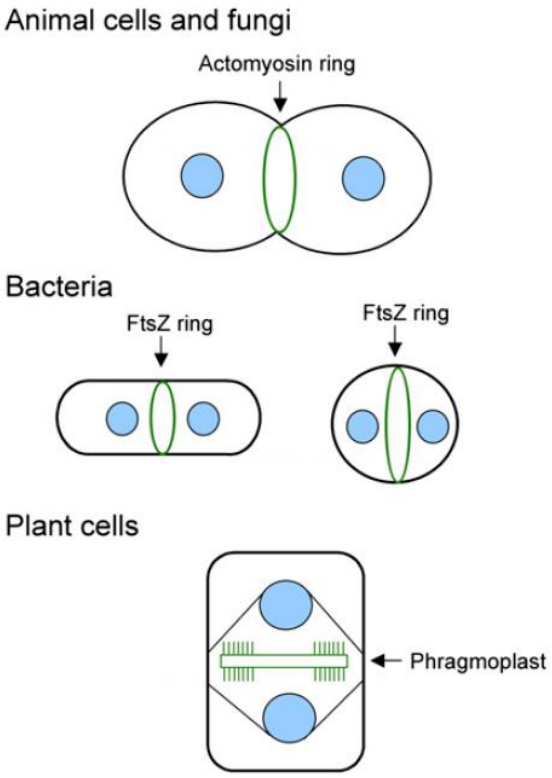


Figure 3: Different mechanisms of division in different organism. (Form (Oliferenko, Chew et al. 2009))

Assembly of cytoskeletal proteins into cytokinetic machineries in different cell types. A contractile ring composed mainly of F-actin and myosin is used for cytokinesis in animal cells and fungi. In bacteria, a tubulin-like protein FtsZ assembles into a ring-structure at the division site. Plant cells use a microtubule-based machinery known as the phragmoplast for cell division

Cytokinesis in fungal amoeboid and animal cells takes place in four steps (figure 4).

- Definition of the division plane
- Recruitment and assembly of the contractile ring components
- Ring contraction and furrow ingression
- Abscission that permanently separate the two daughter cells

First the cell needs to choose the division site; it is an important step since the division plane needs to take into account the spindle axis and other factors in order to avoid cut phenotypes. Once the division site has been chosen, the contractile ring assembles. Its main components are actin cables and myosin II. The third step is the constriction of the actomyosin ring and formation the cleavage furrow. Finally the separation of the daughter cells occurs. In metazoans in happens in a process called abscission and in fission yeast it requires the digestion of the cell wall that separates the two daughter cells (Oliferenko, Chew et al. 2009; Pollard and Wu 2010).

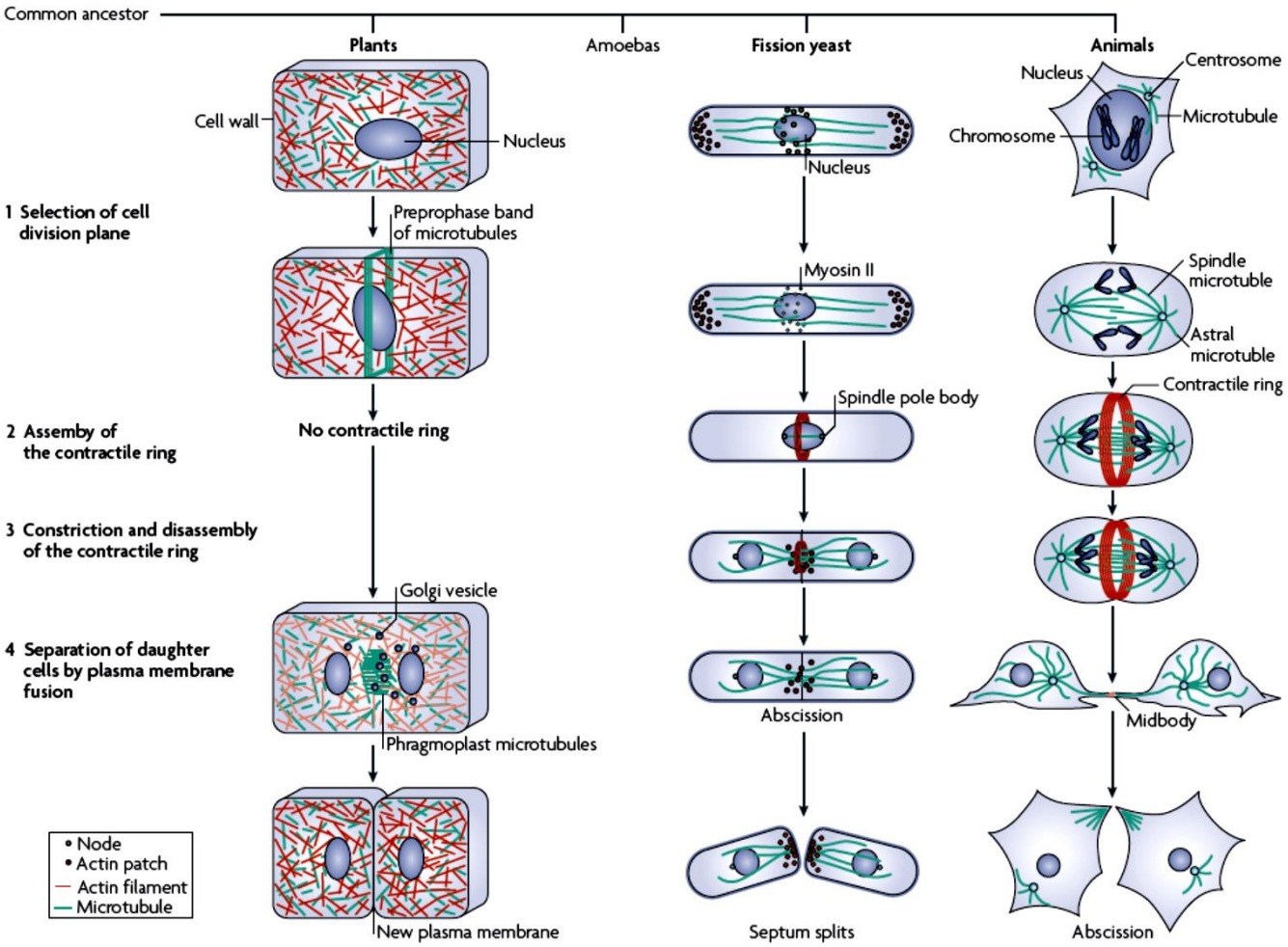


Figure 4: Different steps of cytokinesis in different organisms. (From (Pollard and Wu 2010))
In plants, the cell division plane is selected by the nucleus specifying the position of a preprophase band of microtubules around the equator. Plants lack key proteins to make a contractile ring, so they depend on membrane fusion to separate the two daughter cells. Phragmoplast microtubules transport Golgi vesicles to the midplane to form the new plasma membrane. Amoebas divide much like animal cells and are not illustrated. In fission yeast, the cell division plane is selected by the nucleus specifying the position of nodes around the equator, whereas in animals, spindle and astral microtubules specify the position of the contractile ring. Fission yeast and animal cells assemble a contractile ring of actin filaments and myosin II around the equator of the cell between the chromosomes, which are separated by microtubules of the mitotic apparatus. The ring constricts and the daughter cells separate by membrane fusion.

4. Coordinating growth with division

Cell populations within tissues, or populations of unicellular organisms of the same species, tend to have a similar range of cell sizes suggesting that mechanisms controlling cell size homeostasis exist.

Controlling cell size is indeed very important since the size of a cell influences all the basic physiological cell functions, membrane transport, metabolic flux, biosynthetic capacity, interaction with the environment and nutrient exchange. As a consequence changes in the cell size or cell surface have an impact on these processes. A second reason is that the basic mitotic machinery in eukaryotes needs to be able to operate within the cell. The astral spindle microtubules need to be able to anchor to the cortex in order to position the spindle, and because of the dynamic properties of microtubules they are only able to operate within a certain range. If the cell is too big it may have problems coordinating mitosis and cytokinesis or if it is too small the spindle may not have enough space to assemble and separate the chromosomes. Lastly in multicellular organisms size often is related to function and within a tissue all the cells are the same size.

Cell size homeostasis requires equilibrium between cell growth and cell division. The existence of mechanism that regulates the balance between cell growth and cell division has been postulated decades ago. But despite the importance of the subject no description of molecular mechanism controlling cell size homeostasis has been proposed until recently. The data available until now remain scarce, especially in animal cells, and contradictory. The simplest model to explain size homeostasis would be one in which the cell grows at a constant rate and divides following a cell cycle clock or **timer**. This hypothesis would not require the cells to know how big they are. But good evidence that cells can sense their size or have a **sizer** has in addition been gathered in yeasts.

In fission and budding yeasts, the small variability seen in cell size in a population cannot be explained only by the existence of a timer. Budding yeast cells adjust the time spent in G1 to undergo start at a similar size. Fission yeast cells adjust the time spent in G2 to divide at a fixed cell size. Best evidence that cell size sensors exist comes from experiments where fission yeast cells are arrested in the cell cycle progression and become longer. Upon release of the arrest, the cells undergo shorter cell cycles until the wild type cell length has been recovered, arguing for the existence of cell size sensors (figure 5) (Fantes and Nurse 1977; Fantes 1977; Fantes 1981; Turner, Ewald et al. 2012).

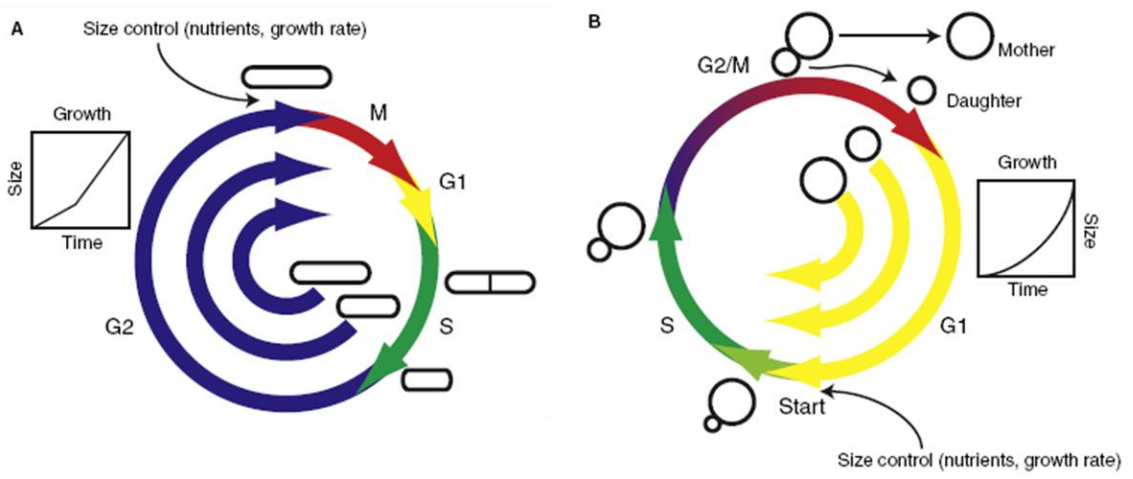


Figure 5. Size-dependent cell cycle progression in S. pombe and S. cerevisiae. (From (Turner, Ewald et al. 2012)
(A) S. pombe cells enter G2 at different sizes following S-phase. They grow in a bilinear fashion and enter mitosis upon reaching a threshold size, so that smaller cells spend more time in G2 than larger cells, as indicated. (B) S. cerevisiae daughter cells are born at different sizes and grow exponentially. Smaller cells spend more time in G1 prior to Start than larger cells (as indicated), which partially compensates for initial size variation. Size control is a function of nutrient conditions and growth rate and is exerted at G2–M in S. pombe and within G1 in S. cerevisiae.

Despite the obvious interest of the cell size homeostasis question, most of the information and knowledge about cell size regulation comes from old studies. And most of it remain descriptive. The main problem that such studies have faced is the lack of a good

methodological approach or technique. Nevertheless, the advances of microscopy techniques combined to mathematical modelling has allowed to perform a series of new studies that support the existence of a cell size sensing mechanism in metazoans, in lymphoblasts (Tzur, Kafri et al. 2009) and HeLa (Kafri, Levy et al. 2013; Sung, Tzur et al. 2013) cells. These studies propose the existence of a cell size sensing mechanism operating at G1/S to reduce the variability of cell size generated by small asymmetries in cell division. Indeed, since growth is exponential, small differences in cell size at birth would lead to important differences in division in absence of mechanism actively regulating cell size. Thus, cell size sensing mechanisms may be universal.

One factor that is known to greatly influence cell size is ploidy. Cell size increases with DNA content and this correlation is observed through living organisms (Gregory 2001; Marshall, Young et al. 2012). This observation indicates that somehow cells can monitor their ploidy and integrate this information into the cell size-monitoring mechanisms.

In general terms, two types of models by which this might operate have been proposed in the literature: either the cell makes a specific amount of a critical component according to ploidy. We can imagine the cell producing this component as a single peak early in the cell cycle, more gene copies leading to a higher production, this component would then inhibit division until it has been diluted enough. In such case, a larger ploidy would translate into a bigger production and a larger critical volume. In the second kind of model, the cell would produce a component that would be measured against ploidy. As an example a cell could produce a protein that is kept at constant concentration and binds certain sites in the genome. As the cell grows, more protein is produced and more sites occupied. The occupancy of these sites could drive cell cycle transitions. With a bigger ploidy, if the protein concentration is constant, more protein would be needed to bind an enlarged number of sites, leading to a bigger cell size (Marguerat and Bahler 2012; Navarro, Weston et al. 2012).

B. Principles of cytoskeleton organization

In a cell, the structural support, transport of molecules, organelle positioning, motility and division are provided by the cytoskeleton. Traditionally we describe three kinds of proteins, able to form cytoskeletal filaments, F-actin, microtubules and intermediary filaments. These three sets of proteins hold the internal structure of the cell in place in a dynamic way. We will now describe the basic features of F-actin and microtubules assembly and organization in functional networks.

1. F-actin networks

a) - Structure and properties of actin filaments

F-actin is the thiner and more flexible of the cytoskeleton components. It forms filaments 7nm wide. The actin filaments assemble from monomers of actin that interact in a head-to-tail polarized manner to form filaments. In yeast, we find only one actin gene, but in human we find 6 actin genes, two expressed in non-muscular cells and 4 expressed in the muscle. Each actin monomer interacts with the previous one twisted 166°. This gives F-actin the aspect of a double helix. Since all actin monomers in the filament are oriented in the same direction this gives a polarity to actin filaments and different properties to each end. The barbed end or + end grows a lot faster than the pointed end or – end. Actin monomers bind ATP that they hydrolyze to ADP after being incorporated to the filament. Although actin can polymerize without ATP, ATP bound actin monomers polymerize a lot faster that monomers bound to ADP. Actin polymerization is a reversible process, therefore actin filaments can depolymerize as actin monomers are removed from filament ends. Since Actin-ADP has a lot less affinity than Actin-ATP, the depolymerization of an actin filament normally occurs at the pointed end, while the polymerization happens at the barbed end. This gives rise to treadmilling of monomers on F-actin filaments (Kueh and Mitchison 2009).

b) Modulation of F-actin dynamics in vivo

The dynamics of actin filaments is regulated by several factors that bind to the filament end or lateral surface. Profilin binds to ATP bound actin monomers and carries them at the barbed end contributing to the treadmilling (Yarmola and Bubb 2006). Another category of factors are the capping proteins. Capping proteins like CapG or Gesolin (figure 8) bind to barbed ends (Schafer, Jennings et al. 1996) which allows the increase of the pool of free actin G available for other barbed ends to use. They also contribute to the density of an actin network by limiting the length of the filaments (Wiesner, Helfer et al. 2003). In contrast, Cofilin binds to the pointed end of an actin filament promoting a fast disassembly; cofilin can also "cut" actin creating more pointed ends to increase the depolymerization speed.

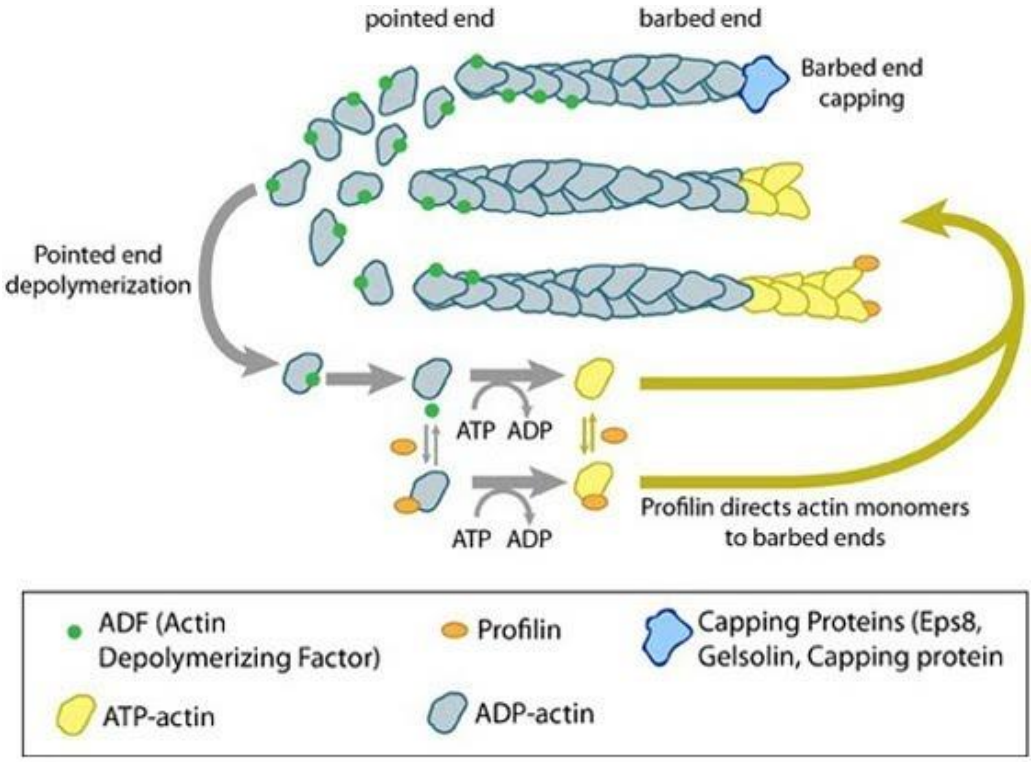


Figure 6: Regulation of actin treadmilling (From: Mechanobiology Institute, Singapore)
The length of actin filaments is controlled by actin binding proteins. Capping proteins prevent assembly at the barbed end while ADF/Cofilin binds to the side of ADP-actin filaments to cause disassembly of the filament. In the absence of actin-binding proteins, the filament length is stable by a treadmilling mechanism (middle filament). Profilin enhances filament assembly by promoting ADP to ATP exchange on actin and by directing actin monomers to the barbed end of filaments (bottom filament).

Nucleation and organization of F-Actin networks

To start actin assembly *in vivo* the cells must generate new barbed ends. But the assembly of the first monomers of a new actin filament is a difficult, rate-limiting process. For this reason cells use actin nucleators. Actin nucleators can make two kinds of structures: branched actin networks and linear actin networks.

Branched actin networks

The best studied actin nucleator for branched networks is the Arp2/3 complex. The Arp2/3 complex is composed of 7 proteins: Arp2 and Arp3 as well as the subunits Arpc1 to Arpc5. This complex is very well conserved through eukaryotes.

In vivo, Arp2/3 is important to organize networks of branched filaments required for endocytosis and phagocytosis or for intracellular vesicle trafficking and creation at the Golgi and ER. Arp2/3 also forms the branched actin network found in motile structures like lamellipodia.

In vitro, the Arp2/3 complex binds laterally to a preexisting actin filament and initiates the assembly of a new filament with an angle of 70° (Goley and Welch 2006). The Arp2/3 complex remains at the boundary between the two filaments unless a recycling mechanism for Arp2/3 is activated. Like the actin monomers the Arp2 and Arp3 subunits of the complex bind to ATP and the activity and conformation of the complex depends on this binding (Goley, Rodenbusch et al. 2004; Zencheck, Xiao et al. 2009). It also seems that the ATP status of the complex plays a role in its recycling (figure 7).

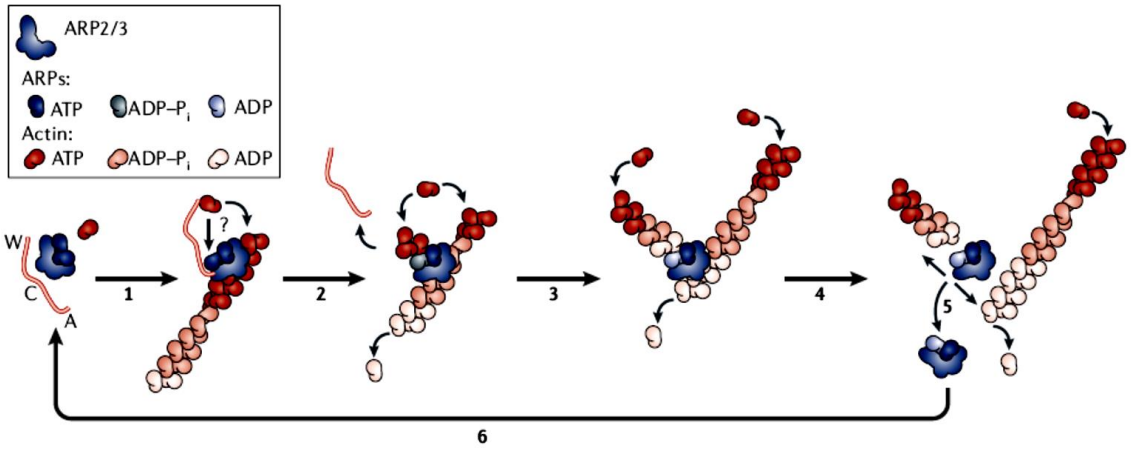


Figure 7: Model for activation and recycling of the ARP2/3 complex. (From (Goley and Welch 2006)

Actin-related protein-2/3 (ARP2/3) complex is shown in blue and actin in red. The nucleotide state of ARPs and actin is indicated by different shading (ARPs: ARP2/3–ATP, blue; ARP2/3–ADP–inorganic-phosphate (Pi), grey; ARP2/3–ADP, light grey. Actin: actin–ATP, red; actin–ADP–Pi, pink; actin–ADP, light pink). The ARP2/3 complex starts in an inactive, open conformation. (step 1) Binding of WCA (Wiskott–Aldrich syndrome protein (WASP)-homology-2, central, acidic) domain promotes a conformational change that primes the complex for activation, which occurs upon binding of the WCA–actin–ARP2/3 assembly to the mother filament, preferentially near the barbed end. WCA domain presents an ATP–actin monomer to the complex and/or possibly to the barbed end of the mother filament. (step 2) ATP is hydrolyzed on ARP2 concomitant with or shortly after nucleation of the daughter filament. The WCA dissociates, although the trigger for this is unknown. (step 3) Phosphate is released from ARP2. Mother and daughter filaments elongate and age by ATP hydrolysis and phosphate release. (step 4) Phosphate release from ARP2 and filament ageing weaken the interactions between ARP2/3 and the daughter and/or mother filament, (step 5) allowing branch disassembly and release of the ARP2/3 complex, presumably in an inactive, ADP-bound conformation. (step 6) Nucleotide exchange on ARP2 (and possibly on ARP3) occurs and the cycle begins again.

Alone the Arp2/3 complex is not a very efficient nucleator and it requires accessory proteins to work: the NPFs (Nucleator-Promoting Factor). In mammalian cells the NPFs activate Arp2/3 thanks to the WCA domain. These domains are constituted of a WH2 domain (WASP Homology 2, W) that binds to actin monomers, as well as an amphipathic region (C) and an acid region (A) both binding to Arp2/3. WCA interacts with the Arp2/3 complex creating conformational changes that render it able to nucleate (Goley, Rodenbusch et al. 2004; Rodal, Sokolova et al. 2005; Zencheck, Xiao et al. 2009) while the WH2 domain binds and provides the actin monomers required for nucleation to start.

Linear actin network

Formins are the nucleators of linear actin. Formins have very conserved domains FH1 and FH2 (Formin Homology Domain). Formins have been mainly studied in yeast although there is fifteen formins in mammalian cells. Formins are responsible for several actin structures from stress fibers to the contractile ring. (Hotulainen and Lappalainen 2006; Watanabe, Ando et al. 2008).

The FH2 domain is sufficient to start nucleation in vitro (Chesarone, DuPage et al. 2010). Unlike Arp2/3 which remains at the pointed end, the FH2 domains act like a processive cap for the barbed end. They block the activity of other capping proteins that may interrupt elongation (Romero, Le Clainche et al. 2004). An example of this is the competition existing in *S. cerevisiae* cells between the formin Bnr1 and the polarity factor Bud14 to regulate the length of actin cables (Chesarone and Goode 2009).

The active form of the FH2 domains is homodimer with the shape of a ring (Harris, Li et al. 2004; Moseley, Sagot et al. 2004; Harris, Rouiller et al. 2006). This homo-dimerization is necessary for the nucleation. At the barbed end the FH2 dimer alternates between two conformations: open and closed. In the closed conformation, the two FH2 domains are linked to the two last actin monomers added to the filament which renders the elongation of the filament impossible. On the contrary the open conformation corresponds to the processive state of the protein that allows the incorporation of G-actin (Otomo, Tomchick et al. 2005).

The nucleation mechanism works in the following way. A dimer of FH2 binds to the barbed end of a filament of actin while the FH1 domains recruits profilin bound G-actin. The domain FH1 provides actin to the barbed end (Otomo, Tomchick et al. 2005; Paul and Pollard 2008). The FH2 domains move with the growth of the barbed end.

The activity of most formins is regulated by auto inhibition, as shown for the drosophila formin Diaphanous. These formins have an N-terminal DID domain (Diaphanous Inhibitory Domain) that participates to the autoinhibition (Li and Higgs 2005), followed by a coil-coil domain (CC) and a dimerization domain (DD) in a central position. The C terminal part has an FH1 and FH2 as well as a DAD domain (Diaphanous Autoregulatory Domain). The interaction between DID and DAD domains inhibit the formin activity (Wallar, Stropich et al. 2006). Binding of small GTPases to the DID domain breaks the DID-DAD interaction and therefore the autoninhibition (Lammers, Rose et al. 2005).

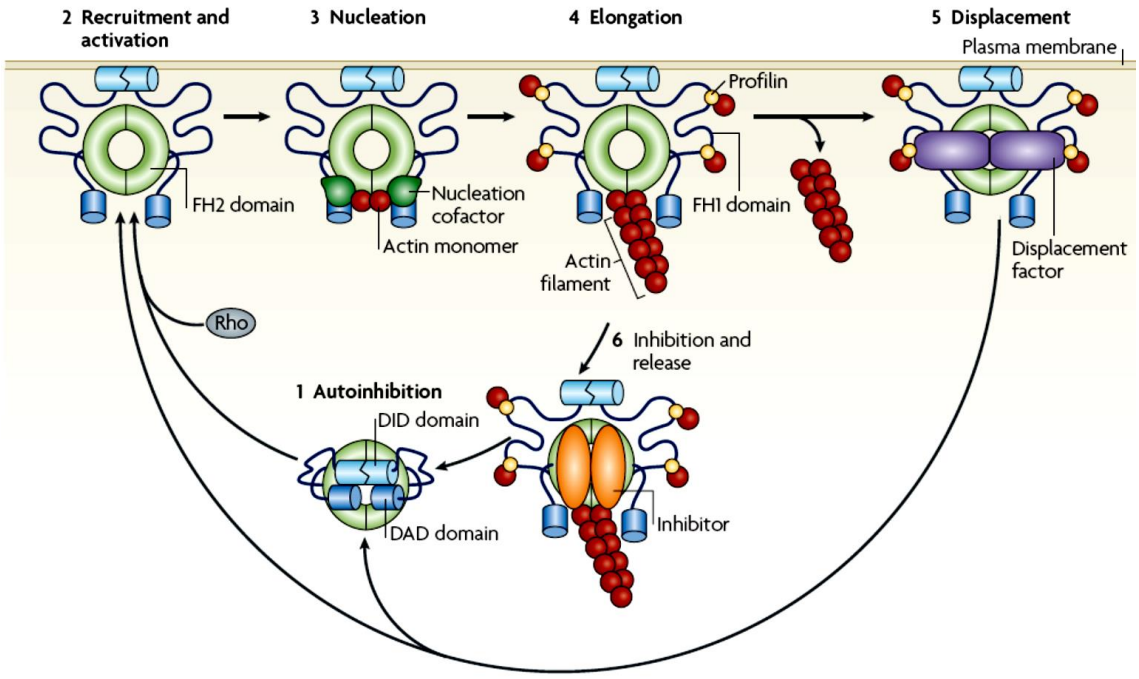


Figure 8: Proposed model of regulatory points in the formin activity cycle. (From (Chesarone, DuPage et al. 2010)

Formin dimers are autoinhibited in the cytosol through interactions of their amino-terminal diaphanous inhibitory domain (DID) and their C-terminal diaphanous autoregulatory domain (DAD) (step 1). Formins are recruited to and activated at the plasma membrane by Rho proteins and possibly other factors (not shown). This leaves the DID associated with the plasma membrane and liberates the doughnut-shaped formin homology 2 (FH2) domain and the adjacent DAD to initiate actin assembly (step 2). Nucleation of an actin filament may involve FH2 binding and the stabilization of transient actin polymerization intermediates (step 3). In some formins, strong nucleation might require an actin monomer-binding nucleation cofactor, such as the one depicted here (based on bud site selection protein 6 (Bud6)), to associate with the DAD. The barbed end of the nascent actin filament is captured by the FH2 domain (step 4). The FH2 stays processively attached to the growing end as new actin subunits are rapidly added. Elongation is accelerated by the flanking, rope-like FH1 domains through their ability to recruit and deliver profilin–actin complexes to the growing barbed end. Interactions between the FH2 domain and a formin displacement factor trigger the release of the actin filament and its incorporation into an actin network, leaving the inhibited formin membrane-associated (step 5). The formin and its attached actin filament jointly dissociate from the membrane (step 6). This is probably coupled to formin inhibition, which is achieved through post-translational modification and/or the binding of additional cellular factors

In conclusion, F-actin can adopt very different types of organization depending on the nucleators they are assembled from and on proteins that associate with the network to modulate the dynamics of the filaments or the architecture of the network. Besides structural support, F-actin networks provide tracks for myosin motors, which are minus end directed that perform vesicular transport or control F-actin networks contractility. Noteworthy, most of the proteins involved in F-actin network regulation are conserved in all eukaryotes including *S. pombe* as will be discussed later in the polarity and morphology section of this manuscript (Kovar, Sirotkin et al. 2011).

2. Microtubule networks

a) -Microtubule structure and properties

Microtubules are the more rigid cytoskeleton component. As the name indicates they are hollow tubes of about 25 nm in diameter. Like F-actin, microtubules are dynamic polymers which assemble and disassemble following the cell needs and depending on which proteins they interact with. Tubulin is a dimer made by two small proteins α-tubulin and β-tubulin. A third kind of tubulin exists, γ-tubulin which plays an essential role at the nucleation of microtubules (see below). Tubulin dimers polymerize to form protofilaments. The microtubule normally consist of 13 linear protofilaments assembled around a hole. The protofilaments, that are constituted by the tubulin dimers in a head to tail fashion, are disposed parallel to each other. As a consequence microtubules, like actin are polar structures with two different ends: a plus end and a minus end.

Tubulin dimers can polymerize and depolymerize quickly. Both α and β tubulin can bind to GTP, which acts in a similar way as the ATP acts on the actin monomer. In fact, it is the GTP bound to β-tubulin, and not to α-tubulin, that gets hydrolyzed during or just after polymerization. This hydrolysis of GTP diminishes the affinity of a tubulin dimer for its neighbors which promotes dissociation and creates the dynamic behavior of the microtubules.

Like ATP-actin, GTP bound dimers bind better and faster at the plus end of the microtubules and the GDP bound dimers tend to dissociate from the minus end of the microtubule. Even though it resembles a lot actin, treadmilling does not occur in microtubules; instead we define the behavior of microtubules in the cell as dynamic instability (Howard and Hyman 2003).

The growth or shortening of microtubules is dictated by the speed of addition of GTP bound dimers relative to the speed of GTP hydrolysis, if the binding is faster than the hydrolysis then the microtubule can maintain a GTP cap and will keep growing, alternatively if the speed of addition decreases, the microtubule will lose its GTP cap and the microtubule will undergo a fast depolymerization called catastrophe. Dynamic instability that was described by Tim Mitchison and Marc Kirschner in 1984, gives rise to a fast and continuous renovation of most of the microtubules (Mitchison and Kirschner 1984). This is for instance essential for the cytoskeleton adaptation to cell shape changes during migration or to the complete remodeling required during mitosis.

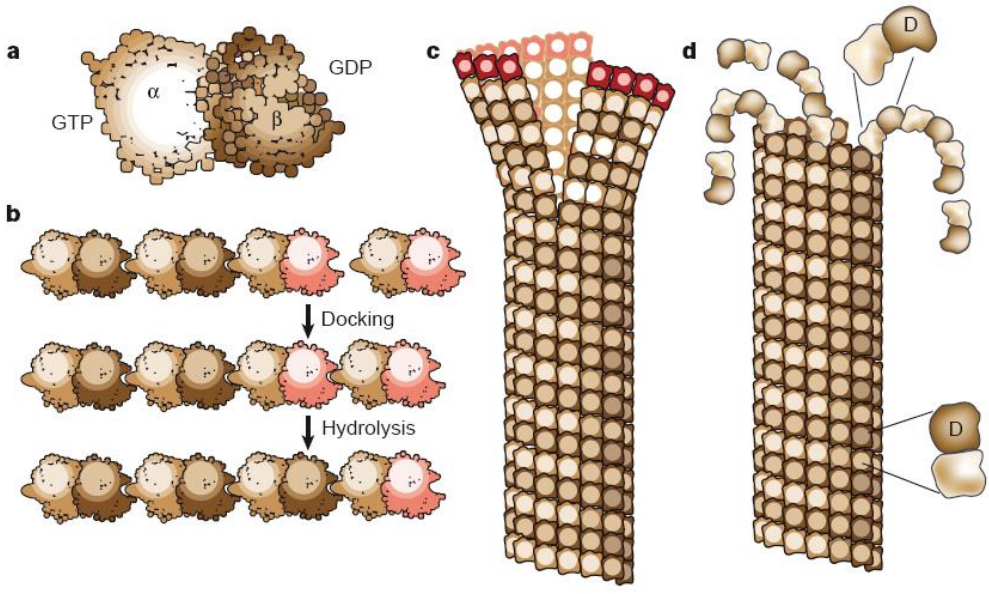


Figure 9: Model for how the GTP hydrolysis cycle is coupled to structural changes in the microtubule. (From (Howard and Hyman 2003))
a, Atomic structure of the tubulin dimer. b, Docking of the a-b subunit to the microtubule end. Residues from the incoming a-subunit trigger hydrolysis of the GTP bound to the lattice attached b-subunit. c, d, Microtubules at growing ends contain sheets of protofilaments while microtubules at shrinking ends curl..

b) Modulation of microtubule dynamics by MAPs

Dynamic instability is linked to particular characteristics of microtubules discussed above but is efficiently modulated by a group of proteins called MAPs (microtubule associated proteins). Within this group of proteins, we find the microtubule associated motors dynein which walk towards the minus end of microtubules, and kinesins which walk towards the plus end of microtubules. A particularly important family of MAPs for microtubule dynamics are the + TIP proteins. + TIPS associate specifically with the growing end of microtubules (Schuyler and Pellman 2001). This family includes EB1 and EB3 that promote the stable and continuous growth of microtubules by preventing catastrophe. We also find the CLASPs proteins (Cytoplasmic Linker Protein-Associated Proteins), that stabilize microtubules at the level of the cell cortex (Mimori-Kiyosue, Grigoriev et al. 2005), the CLIPs (Cytoplasmic Linker Proteins) which promote microtubule rescue in vivo (Komarova, Vorobjev et al. 2002; Mimori-Kiyosue, Grigoriev et al. 2005), or XMAP215, a MAP identified in Xenopus which increases the speed of microtubule polymerization (Vasquez, Gard et al. 1994; Tournebize, Popov et al. 2000).

c) Microtubule nucleation and organization by MTOCs

Microtubule nucleation by the γ-TURC

In most cells microtubules are anchored by their minus end to an MTOC (microtubule organizing center) from which they are nucleated. In animal cells the main MTOC is the centrosome which is attached to the nuclear envelope in interphase cells (Bornens 1977) and composed of two centrioles and pericentriolar material (PCM) that contains microtubule nucleators.

Indeed, similar to F-actin, a rate-limiting step for microtubule assembly is the nucleation of new microtubules from αβ-tubulin dimers. This step is favored *in vivo* by complexes organized

by γ-tubulin, a rare form of tubulin conserved among eukaryotes. To form nucleation complexes γ-tubulin associates with GCPs (for γ-tubulin complex proteins). Two different complexes have been identified: the γ-tubulin small complex (g-TuSC), which is composed of two molecules of γ-tubulin associated with one molecule of GCP2 and GCP3, and the γ-tubulin ring complex (γ -TuRC), which resembles a ring. The γ -TuRC is composed of multiple copies of γ -TuSC proteins and several additional proteins

Different mechanisms for nucleation based on these two structures have been proposed: the template model and the protofilament model. The template model is the most accepted one and it proposes that the αβ-tubulin dimers interact with the ring like shape of the γ-TuRC through the α-tubulin which would face the minus end of the newborn microtubule. The new microtubule would then grow using this base as a template. The protofilaments model proposes that the tubulin dimer would interact laterally with the open end of the γ-TuRC. According to this model, the γ-TuRC would not be a template ring but a protofilaments that curves due to the intrinsic curvature of the monomers interaction (figure 10) (Raynaud-Messina and Merdes 2007).

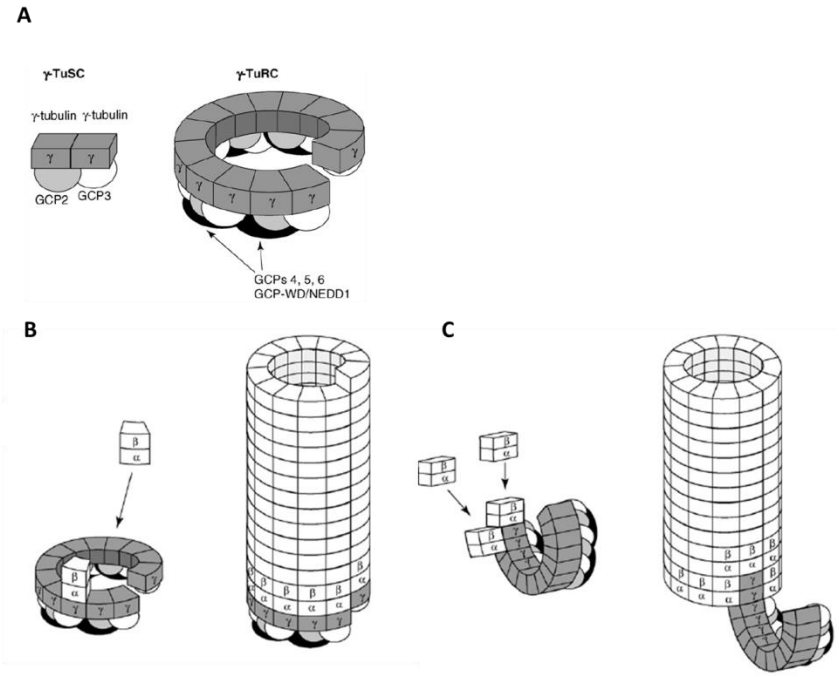


Figure 10: Schemes of γ-tubulin complexes and nucleation models. (From (Raynaud-Messina and Merdes 2007))
(A) Schematic drawings of a γ-tubulin small complex (γ -TuSC) and a γ-tubulin ring complex (γ -TuRC). The structure of the γ -TuRC resembles a ring or a lock washer, as seen by electron microscopy.
Potential mechanisms of γ-TuRC-induced microtubule nucleation. (B) Template model: γ-tubulin molecules (grey) associate laterally to form a ring or lock-washer-shaped complex. The number of γ-tubulin molecules in the γ-TuRC defines the number of protofilaments in the microtubule. α/β-tubulin dimers are arranged such that b-tubulin is oriented towards the plus end. (C) Protofilament model: the γ-TuRC consists of longitudinally associated γ-tubulin molecules (grey) that produce a curled or lock-washer-shaped structure because of the curved conformation of each γ-tubulin monomer. Dimers of α and β-tubulin can associate laterally and longitudinally to form protofilaments that close into a microtubule.

In vitro polymerized microtubules can have from 11 to 16 protofilaments, but *in vivo* they almost always have 13 protofilaments. This fact supports the template model since the template ring has 13 subunits. Also, several studies have recently provided structural data which has confirmed the template model (Kollman, Merdes et al. 2011). It is still in question if both models coexist or if only the template model accounts for all nucleation events in living cells.

Organization of microtubule networks by MTOCs

In most cells, the dynamic plus end of microtubules is oriented towards the periphery of the cell while their minus end is anchored at centrosomes where nucleation took place. The γ-TuRC that remains attached to the minus end would have a protective role, preventing depolymerization.

Interestingly, some cells have secondary MTOCs which accumulate γ-tubulin outside of the centrosome. A good example of γ-tubulin accumulation outside the centrosome is the spindle. In the spindle γ-tubulin is not only present at the spindle poles but also in the kinetochore microtubules during metaphase and anaphase but also in the spindle midzone during anaphase (Mastronarde, McDonald et al. 1993; Buster, McNally et al. 2002).

In conclusion, thanks to the different kinds of organization they can adopt and to their ability to be remodeled quickly, microtubule networks have lots of different cellular functions. For instance, they provide the axonal guide in neurons, the tracks for the vesicular transport between different cell compartments and make the mitotic spindle for chromosome segregation during mitosis.

II.
Introduction

II. Introduction

A. *Schizosaccharomyces pombe* as a model in cellular biology

1. Model presentation

a) Origins of fission yeast and selection as a model organism

The fission yeast *Schizosaccharomyces pombe*, was first isolated from East African beer by the German biologist P. Linder. But the use of fission yeast as an experimental organism was initiated later by Urs Leupold in the 1940s. He developed the initial genetic studies and selected *wt* homotallic (h90) and heterotallic (h+ and h-) strains used in all the fission yeast labs around the world. From these strains, the rest of worldwide lab collections have been developed. In the 1950s Murdoch Mitchison realized the potential of this organism for studies of cell physiology and began to analyze the cell growth during the cell cycle.

Ever since fission yeast has attracted geneticists and biologists that were seduced by the easy genetic manipulations that could be done with this yeast. Most importantly the field of cell cycle research took off in the early 1970s, when Paul Nurse, after spending several months in the lab of Urs Leopold, went to Mitchison's lab in Edinburgh and started the ***cdc*** screens (*cdc* for cell division cycle).

This work allowed Paul Nurse and his collaborators (Nurse, Thuriaux et al. 1976; Nurse and Thuriaux 1980) to identify the main components that control the biochemical clock of the cell cycle like the cyclin dependent kinase Cdc2 (Cdk1), the cyclin Cdc13 (Cycline B), or Cdc25 phosphatase, and develop the initial model of cell cycle control in fission yeast, which will be detailed later on.

The first *cdc* screens also allowed to identify numerous cytokinesis mutants (*cdc3*, *cdc4*, *cdc7*, *cdc8*, *cdc11*, *cdc12*, *cdc14*, *cdc16*). The study of these has allowed since the beginning of the 90s a thorough dissection of the molecular mechanisms of cytokinesis. Other screens followed

that allowed the identification of about 130 genes involved in cytokinesis (Gould and Simanis 1997; Guertin, Trautmann et al. 2002). The mechanisms of cytokinesis in fission yeast will be explained in detail latter on this manuscript.

As a consequence, fission yeast has become one of systems where cytokinesis has been studied in most details (Bathe and Chang 2010; Laporte, Zhao et al. 2010; Pollard and Wu 2010; Goyal, Takaine et al. 2011; Lee, Coffman et al. 2012; Rincon and Paoletti 2012). Since the contractile ring assembly mode and the contraction resemble those of animal cells, these discoveries have paved the way for cytokinesis studies in metazoans.

Finally another set of mutants was discovered during the first genetic screens done in fission yeast: the morphogenesis mutants. Some mutants affect the number of microtubules or their actin dynamics and so they develop misshapen morphologies. We will discuss them latter in the morphogenesis section (Verde, Mata et al. 1995; Verde 1998).

The sequencing of the fission yeast genome finished in 2002, and it revealed 4940 protein coding genes. Genes important for eukaryotic cell organization were found with a high conservation, including those required for the cytoskeleton, compartmentalization, cell-cycle control, proteolysis, protein phosphorylation and RNA splicing. Fifty genes were found to have significant similarity with human disease genes; half of these are cancer related (Wood, Gwilliam et al. 2002). More recently, a deletion collection was made, covering 98% of the fission yeast genome and is now accessible to the community (Kim, Hayles et al. 2010).

b) Fission yeast growth pattern and division cycle

Fission yeast has become over the years a very successful model in cell biology for several reasons. Fission yeast is an easy to grow organism. It is a haploid single cell model which, in combination with a high rate of homologous recombination, makes its genome manipulations

easy. In particular, with the availability of fluorescent proteins in the 90s, has allowed the fast development of powerful live cell imaging approaches.

Fission yeast cells also have a very simple morphology and a reproducible growth pattern and coordinate cell growth and cell cycle in a very robust manner (see next paragraph). This property has allowed the identification in genetic screens such as the *cdc* screen mentioned earlier, lots of mutants affecting cell morphogenesis and cell division, whose characterization has initiated the molecular understanding of these key cellular events.

An exponentially growing newborn fission yeast cell is a small rod that measures 4µm wide and around 7-8µm in length. Like bacteria and plants, fission yeast cells have a rigid cell wall. At the beginning of the cell cycle cell growth is restricted to one end, the old end preexisting in the mother cell. As the cell cycle progresses in early G2 and the cell reaches around 9.5µm, at 0.34 of the cell cycle, the activation of growth in the new end occurs in a process called NETO (for new end take-off). Wild type cells show two linear segments of cell growth during the first 75% of the cycle stopping growth during mitosis, which occupies about 25% of the cell cycle. There is a rate-change point (RCP) that coincides with NETO and that causes a 35% increase in cell rate growth. This increase is not only due to the start of cell growth, since the old end slows down its growth rate after NETO (Mitchison and Nurse 1985). A recent report challenges this view and claims that fission yeast growth is exponential and with no RCPs (Cooper 2013). More work to clarify the situation is required, but so far the linear model is the only one used in all cell cycle studies in *S. pombe*.

When the cells reach 14µm they stop growing and undergo mitosis. As mitosis proceeds an intranuclear mitotic spindle assembles to segregate chromosomes, along with a contractile acto-myosin ring for cytokinesis. The contractile ring always assembles in the middle of the cell to produce two daughter cells of identical size. Contraction of this ring starts at the end of Anaphase B and occurs concomitantly with the synthesis of the septum, the new cell wall that

will separate the two daughter cells. The two daughter cells are finally separated by the action of glucanases.

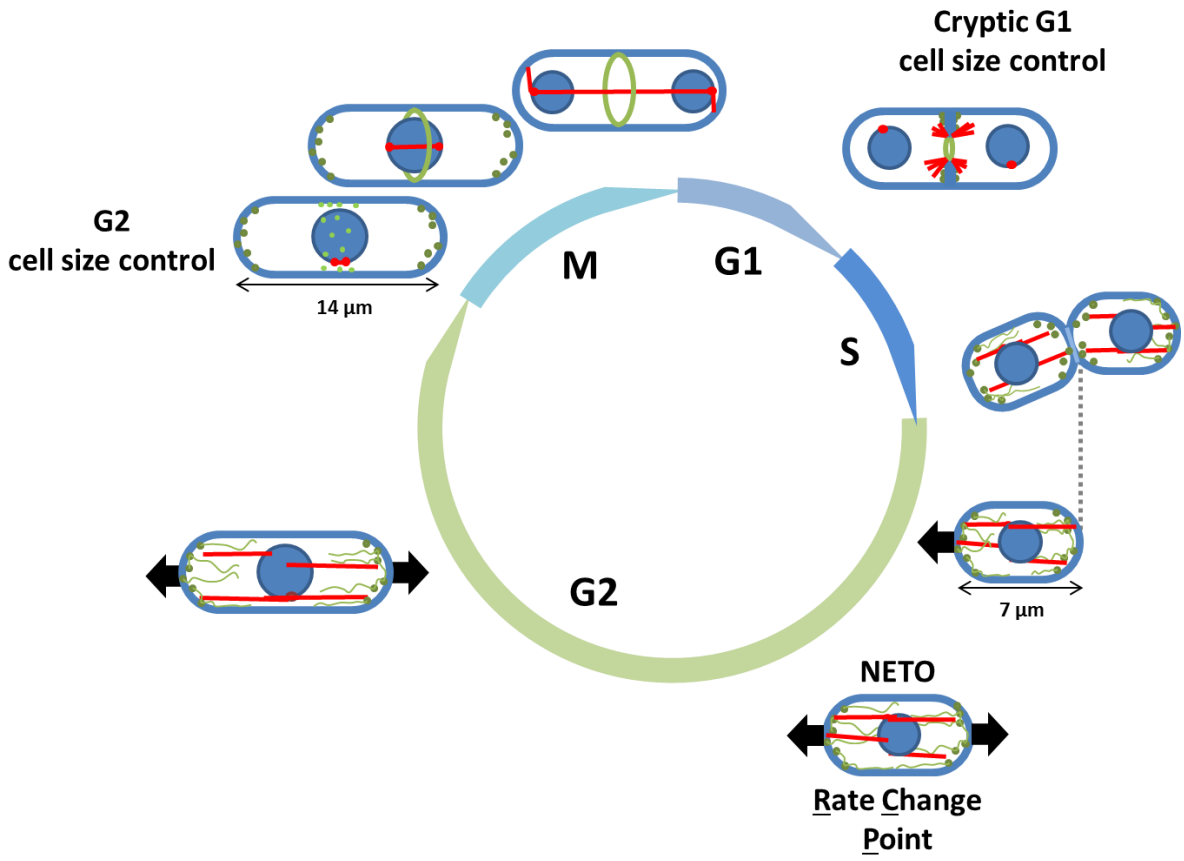


Figure 11: Polarized cell growth during the fission yeast cell cycle.
Newborn fission yeast cells measure 7µm and grow in a monopolar fashion by tip extension using its old end. At approximatively one third of the cell cycle and after having reached 9.5µm in length the cell will activate growth in its new end in a process called NET (new end take-off). The cell will keep growing in a bipolar fashion until it reaches 14µm and enters into mitosis. A cytokinesis actomyosin contractile ring will be assembled at the middle of the cell. The ring contraction will be concomitant with the septum building that will be digested later on and so promote a separation of the two daughter cells.

c) Principles of cell size homeostasis in fission yeast

As mentioned earlier, newborn cells double their size before entering the next round of cell division. Due to the regular rode shape, this doubling in cell size corresponds to a doubling in length. This tight coupling between growth and the cell cycle is the result of cell size homeostasis mechanisms that where described in classic articles in the 70s.

The first evidence for cell size sensors in fission yeast came from cell cycle mutants that were arrested in cell cycle progression and became longer. When the cell cycle block was released, cells underwent shorter cell cycles until the wild type cell length was recovered, arguing for the existence of cell size sensors (Fantes 1977).

Fantes and Nurse further showed that cells large at birth grew less in size and had a shorter G2 phase than the average while cells born short grew more had a longer G2 phase (Fantes and Nurse 1977). This cell size control mechanism thus works by altering the length of the cell cycle rather than by modifying the growth rate (Fantes and Nurse 1977; Fantes and Nurse 1978).

Nurse and Thuriaux realized that besides this G2 cell size control there was another cryptic cell size control in G1. When using *wee1* mutants which have no G2 cell size control they realized of the existence of this G1 control (Nurse and Thuriaux 1977). Normally this control is not used in *wt* cells since they are already too big when they are born, but *wee1* mutants are so short that they delay entry in S phase due to this G1/S cell size control mechanism.

This early work also postulated that cell size homeostasis relied on a combination of timers that set the time it takes to accomplish specific cell cycle steps, and sizers controlling the period of growth by monitoring cell size. According to these classic models, a sizer would be operating during G2 to delay entry in mitosis until the cells reach 14µm.

Cell size homeostasis principles were revisited more recently by (Sveiczer, Novak et al. 1996). These authors found that cells born short extend the cell cycle in length proportionally to their

birth size, but that above a size threshold, larger cells are unable to do reduce their cycle length and divide at a longer cell size than expected. They proposed that a sizer may operating during the first part of the cell cycle until mid-G2, followed by a timer in late G2 that may be necessary to prepare mitotic entry. The change point between sizer and timer would happen just before RCP2 and NETO.

This study also confirmed that *wee1* deficient cells that halt the cell cycle in G1/S instead of G2/M exhibit homeostatic properties and extend their cell cycle length proportionally to their birth size. This shows that the cryptic G1/S size control functions as a sizer and not a timer. Finally, this study shows that upon inactivation of both Rum1 and Wee1 that operate the G1/S and G2/M arrests respectively cells then divide at shorter and shorter cell sizes. This indicates the cell size control is abolished in these conditions and suggests that the two cell size control mechanism can compensate for one another to maintain fission yeast cell size constant.

2. Polarity and morphogenesis in *S. pombe*

Cell morphogenesis is a complicated process that permits cells to adopt specific shapes, adapted to their function. A key step for cell morphogenesis is polarity establishment, under the control of the cytoskeleton. Unicellular organisms provide simplified systems to dissect fundamental aspects of cell morphogenesis. As mentioned earlier, fission yeast, with its regular shape, easy genetics and relatively simple cytoskeleton, has emerged in the 90s as a powerful system to study cell morphogenesis (Verde 1998; Martin 2009; Hachet, Bendezu et al. 2012).

a) *Cytoskeleton organization in fission yeast*

The microtubule cytoskeleton of fission yeast

During interphase, the microtubule cytoskeleton of fission yeast is organized in 3-5 microtubule bundles (Tran, Marsh et al. 2001). These microtubule bundles are composed of antiparallel microtubules overlapping at their – ends at the level of the centrosome equivalent, called the SPB, or at interphase MTOCs (iMTOCs) associated with the nuclear envelope. Microtubule + ends point towards the cell tips (figure 13) and, like in animal cells, exhibit dynamic instability and frequently transition from growth phases towards the cell tip to shrinking phases towards the SPB or iMTOCs (Sawin and Tran 2006).

A model for the formation of these bundles has been proposed. A first microtubule is nucleated from γ-TURCS attached to the nuclear envelope at an iMTOC site through the centrosomin related protein Mto1 and its partner Mto2. Mto1/Mto2 can also recruit the γ-TURCS on pre-existing microtubules to nucleate additional microtubules that adopt an anti-parallel conformation upon binding of the Prc1-like antiparallel microtubule bundler Ase1. Minus ends are grouped in middle by action of Klp2 kinesin that binds to plus ends of

microtubules and slides the new born anti-parallel microtubule towards the iMTOC (Janson, Setty et al. 2005; Loiodice, Staub et al. 2005; Janson, Loughlin et al. 2007).

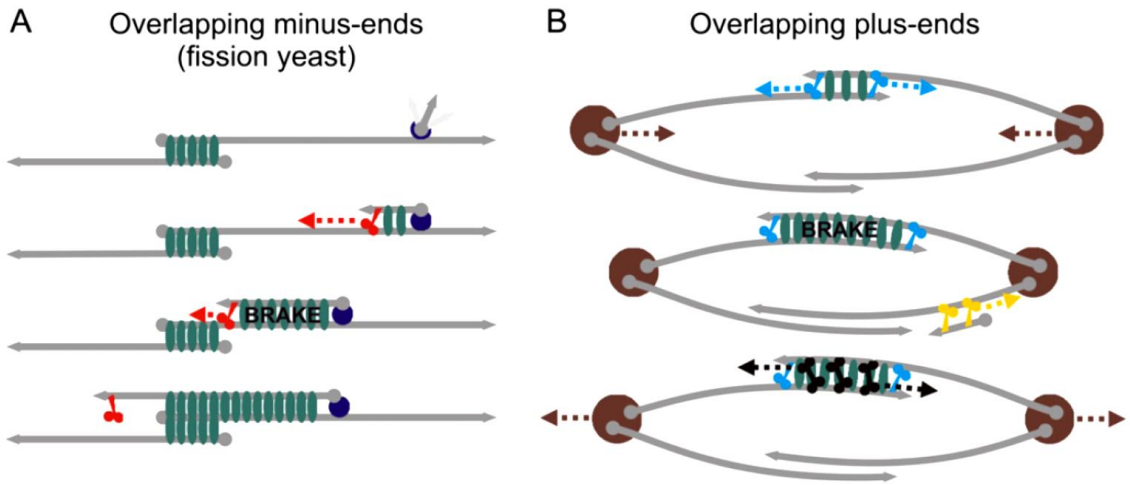


Figure 12: Model for MT Organization with Competing Sliding and Friction Forces. (From (Janson, Loughlin et al. 2007))
(A) MT plus ends are indicated by arrow heads, minus ends by spheres. MT nucleation along interphase bundles occurs from MT-bound nucleation complexes (purple). After nucleation, MTs are stabilized in the antiparallel configuration by polarity-specific ase1p (green). The minus-end-directed kinesin-14 klp2p (red) subsequently transports MTs to the bundle midzone. As the new MT grows, additional ase1p binds, increasing the friction against a length-independent number of motors at MT plus ends. Consequently, the speed of transport decreases and finally becomes zero when motors lose contact with antiparallel MTs.
(B) Possible mechanisms, based on length-dependent and -independent forces, for the regulation of overlap between antiparallel overlapping MT plus ends. The bundling of antiparallel MTs by ase1p (observed in anaphase spindles) would not interfere with the focusing of parallel MTs toward the spindle poles by minus-end-directed motors (yellow). Other minus-end-directed motors may specifically bind to MT plus ends (blue) and pull poles together with an overlap-independent force. An increase in overlap recruits additional ase1p proteins, generating friction that resists MT sliding and slows down pole-to-pole motion. Plus-end-directed motors (black) may bind in a length-dependent manner along MTs, a process potentially regulated by the binding of motors to ase1p. For large overlap, enough plus-end-directed motors may bind to push the poles outward. An equilibrium overlap can then exist at which forces generated by plus- and minus-end-directed motors and ase1p are balanced.

Importantly, interphase microtubules are not required for growth but mutants with defective microtubules properties tend to lose the rod shape and become bent or display branches, indicating that microtubules contribute to proper definition of the growth zones. This is the case of the *tea1* mutants, which are curved and become branched at high temperatures adopting a "T" shape (Mata and Nurse 1997). Other mutants like *tea4* (Martin, McDonald et al. 2005), *mod5* (Snaith and Sawin 2003), *tea2* (Browning, Hayles et al. 2000), *tip1* (Brunner and Nurse 2000), *mal3* (Beinhauer, Hagan et al. 1997) and *pom1* (Bahler and Pringle 1998) have similar morphological defects.

A complete remodeling of the microtubule network takes place at mitotic entry. Interphase bundles disassemble while the mitotic spindle forms, organized by the duplicated SPBs. Like the centrosome in metazoans, the fission yeast SPB spends most of interphase in the cytoplasm attached to the nuclear envelope, where it duplicates in a conservative mechanism (Ding, West et al. 1997). As the cell enters mitosis, the nuclear envelope opens to form a fenestra, where the duplicated SPBs still linked by a bridge insert. Intranuclear microtubule nucleation is then activated. Recent studies have shown the activation and insertion of the SPB into the nuclear envelope is promoted by Cut12 which controls the local activation of Cdk1 at the spindle poles (Tallada, Tanaka et al. 2009; Grallert, Chan et al. 2013). This step also involves Pcp1 that recruits Plo1, contributing to mitotic entry and the γ-TURC that nucleates spindle microtubules (Fong, Sato et al. 2010). The composition of the nuclear envelope may be remodeled by Brr6 to allow SPB insertion (Tamm, Grallert et al. 2011). These steps are followed by the assembly of the bipolar spindle which requires the kinesin Cut7 related to mammalian Eg5, necessary for the interdigitating of the microtubules nucleated by the two SPBs. Through metaphase, the SPBs remain in their fenestrae, bound to the polar ends of spindle MTs. At about this time, a small bundle of cytoplasmic astral MTs forms in association with each SPB a bundle that helps orienting the spindle in the cell (Tolic-Norrelykke, Sacconi et

al. 2004). As anaphase proceeds, the nuclear fenestrae closes, and the SPBs are extruded back into the cytoplasm (Ding, West et al. 1997).

Spindle elongation during anaphase is promoted by the antiparallel microtubule bundler Ase1 in association with the kinesin Klp9. During metaphase, the mitotic kinase Cdk1 prevents their association. In anaphase however, the phosphatase Clp1 dephosphorylates the two proteins which can then associate and localize to the central spindle in between the two sets of chromosomes where they promote microtubule sliding and contribute to spindle elongation (Fu, Ward et al. 2009).

At the end of mitosis a new set of microtubules called PAA (Post Anaphase Array) is nucleated in the equatorial plane by a specific MTOC, the eMTOC (equatorial MicroTubules Organizing Center) attached to the contractile ring through the atypical myosin Myp2 (Hagan and Hyams 1988; Heitz, Petersen et al. 2001; Samejima, Miller et al. 2010). Once the mitotic spindle disassembles and microtubules start forming on the outer face of the SPBs, the eMTOCs disassemble and the iMTOCs reassemble on the nuclear envelop (Zimmerman, Tran et al. 2004).

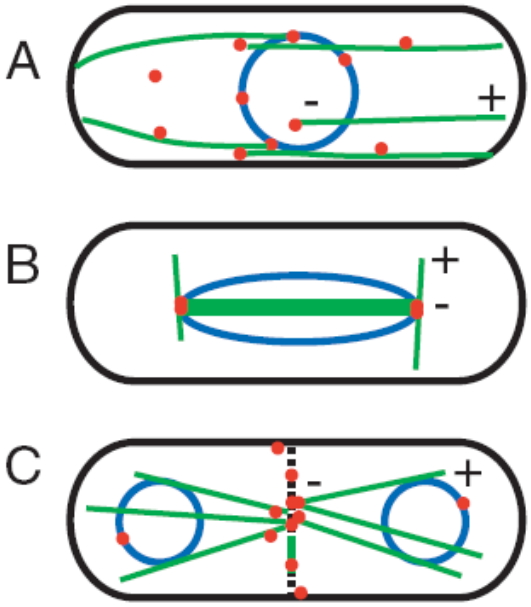


Figure 13. Microtubule organization in the fission yeast cell cycle. (From (Sawin and Tran 2006)
A highly schematic illustration of microtubule (MT) distribution (green) in relation to microtubule organizing centers (MTOCs; red) and the nuclear envelope (blue). During interphase (A), MTOCs may be associated with the nuclear envelope or with existing MTs and may occasionally also be found free in the cytoplasm. MT minus-ends ('–') are generally found towards the cell center and MT plus-ends ('+') towards cell tips. During mitosis (B), intranuclear MTs form the mitotic spindle and astral MTs are nucleated from the SPBs. At the close of mitosis, during cell division (C), the equatorial MTOCs forms at the division site, to nucleate post-anaphase array MTs.

The actin cytoskeleton in fission yeast

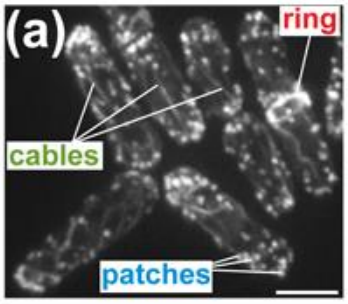
During the firsts screenings in *S.pombe* a set of morphogenetic mutants was found to affect the actin organization and localization at the tips of the cells which in turn lead to round shaped cells, such is the case for mutants like *orb3* or *orb6* (Verde, Mata et al. 1995; Verde, Wiley et al. 1998).

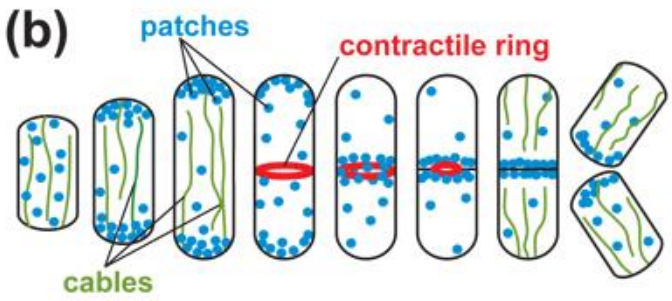
The actin cytoskeleton of *S.pombe* adopts 3 kinds of organization with very specific functions: actin patches for endocytosis, actin cables for vesicle transport and the contractile actomyosin ring for cytokinesis (Kovar, Sirotkin et al. 2011).

Actin patches are nucleated by the Arp2/3 complex (Pelham and Chang 2001). They play a role in polarized growth and are found in the old end only in newborn cells that grow monopolarly and at both the old and new ends after NETO when growth becomes bipolar. Actin patches are associated with endocytosis sites and participate in membrane recycling during cellular growth. During mitosis, the actin patches disappear from the tips and move close to the division site which suggests that endocytosis accompanies septum synthesis. However, the identity of cargos endocytosed at actin patches in fission yeast is not known.

Actin cables are linear actin bundles composed of short parallel actin filaments polymerized by the formin For3 during interphase (Feierbach and Chang 2001; Kovar, Sirotkin et al. 2011). The actin cables provide polarized tracks for type V myosin-directed delivery of vesicles to the growing cell tips for polarized growth. For3 localizes at the cell tips with its activators Bud6 and profilin (Martin and Chang 2006). Cdc42 also participates in the activation of For3 (Martin, Rincon et al. 2007). Fission yeast actin cables are a wonderful example of cytoskeletal crosstalk, since their assembly site is initially established by microtubules as detailed later (Martin 2009).

Contractile actomyosin ring. The description of this structure and its regulation is discussed in details later, in the cytokinesis section.





Actin Structure	Nucleation Factor	Actin Organization	Myosin Motor	Cellular Role
Patches:	Arp2/3 Complex	branched network	Myo1 (type-I)	Endocytosis
Ring:	formin Cdc12	unbranched anti-parallel bundles	Myo2, Myp2 (-II) Myo51 (-V)	Cytokinesis
Cables:	formin For3	unbranched parallel bundles	Myo52 (-V)	Polarized transport

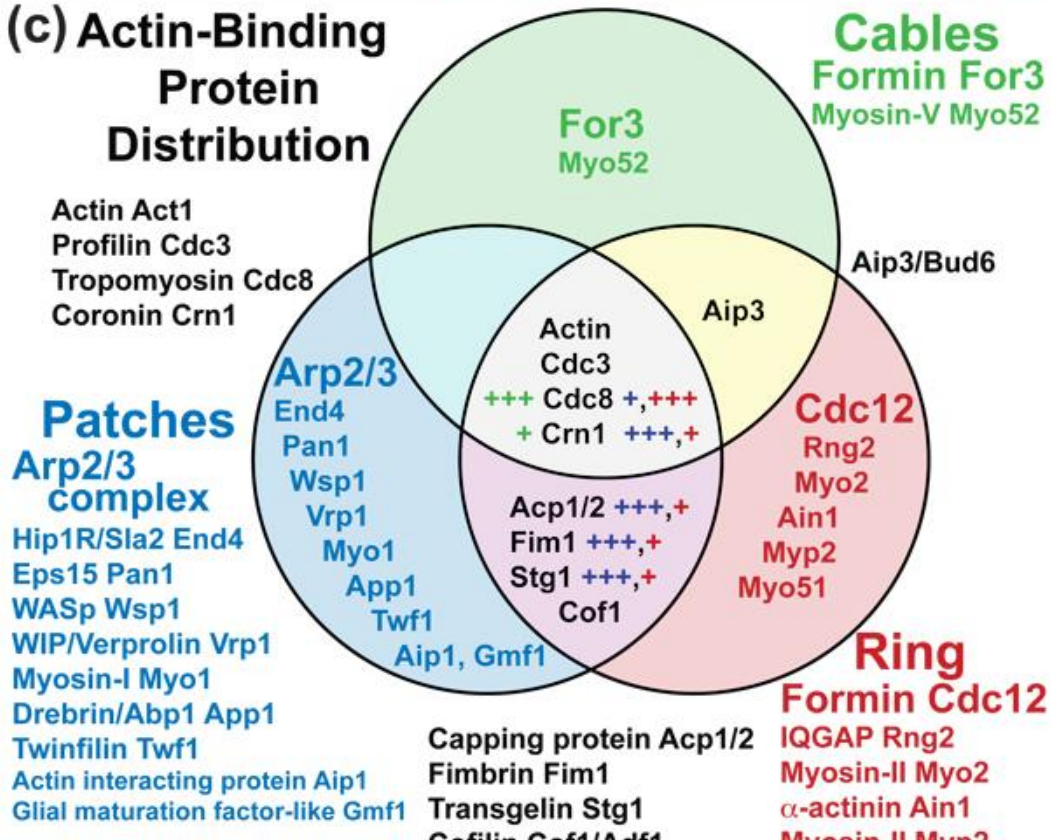


Figure 13. Overview of the three major actin structures in fission yeast. (From (Kovar, Sirotkin et al. 2011))
(a) Fluorescent image of the actin cytoskeleton in a population of fission yeast cells expressing the general F-actin marker GFP-CHD (calponin homology domain of Rng2). (b) Cartoon summarizing the subcellular distribution of actin structures during the cell cycle (centering on mitosis). The table below highlights the basic features and roles of the three actin structures. (c) Venn diagram summarizing the localization of highly conserved actin binding proteins across actin patches (blue), actin cables (green) and the contractile ring (red). Actin-binding proteins are listed under generic and fission yeast protein names in groups outside the diagram based on their cellular distribution. Within the diagram, proteins overlapping two or more structures (black text) are further categorized using colored + signs to emphasize relative protein levels in each actin structure. Arp2/3 complex: consists of seven different subunits. Capping protein: heterodimer of Acp1 and Acp2. Hip1R: Huntingtin interacting protein-related, talin-like. Myosins and IQGAP associate with light chains (Myo1, calmodulin and Cam2; Myo2 and Myp2, Cdc4 and Rlc1; Myo51, calmodulin and Cdc4; Myo52, calmodulin; Rng2, calmodulin and Cdc4). (d) Regulation of actin filament turnover and myosin motors by tropomyosin and fimbrin. Actin patches: High concentrations of fimbrin Fim1 prevent tropomyosin Cdc8 from binding the Arp2/3 complex-nucleated branched filaments, which allows efficient cofilin Cof1-mediated actin filament turnover and recruitment of myosin-I Myo1. Actin cables: tropomyosin favors myosin-V Myo52-directed motility on formin For3-nucleated straight parallel filaments. Contractile rings: Lower concentrations of fimbrin allow limited cofilin severing by partially inhibiting tropomyosin. Tropomyosin also favors myosin-II Myo2-mediated compaction of the formin Cdc12-nucleated straight antiparallel filaments.

b) Polarity and morphogenesis in fission yeast

The rod shaped cell of fission yeast is encased in a cell wall. Its growth is restricted to the cell tips and requires cell wall remodeling helped by turgor pressure. As explained earlier in this manuscript, the growth zones are cell cycle regulated with newborn cells growing only from the old end until the new end is activated in a process called NETO in early G2. Later, when cells enter into mitosis, cell growth stops at the tips and the growth machinery relocalizes at the division site in order to build a septum. These cell cycle-dependent modifications of the growth pattern are linked to changes in cell polarity, under the control of the Rho GTPase Cdc42 which localization and activation dictates cell polarity by influencing the actin cytoskeleton to control cell morphogenesis (Hachet, Bendezu et al. 2012).

Cdc42 GTPase

As all small GTPases, Cdc42 can adopt a GTP-bound active state and a GDP-bound inactive state. The active form of Cdc42 localizes at the cell tips and recent reports have shown that the active form of Cdc42 oscillates between the two cell tips (Das, Slaughter et al. 2012; Das, Drake et al. 2012).

GEFs (guanine nucleotide exchange factors) and GAP (GTPase-activating protein) help switching between this two states and thus control the activity of Cdc42. Scd1 is a GEF for Cdc42 that localizes at the cell tips through the scaffold Scd2. Cdc42 main GAP is Rga4 which localizes at the lateral cortex of the cell. It is the interplay between these activators and inhibitors that regulates the area of activated Cdc42 and determines the standard width of the cell (4µm) (Das, Wiley et al. 2007; Tatebe, Nakano et al. 2008; Perez and Rincon 2010; Kelly and Nurse 2011).

The GEFs and GAPs regulating Cdc42 are in turn regulated by the MOR pathway (Morphogenesis Orb6 Network), a kinase cascade whose downstream component is the NDR kinase Orb6 which inactivation leads to isotropic instead of polarized cell growth (Verde, Wiley

et al. 1998; Kanai, Kume et al. 2005). Despite the importance of this pathway, it is not yet fully understood how it works. One identified action of the MOR is to prevent the lateral localization of Gef1 that serves as a GEF of Cdc42 (Das, Wiley et al. 2009).

Cdc42 effectors

To promote polarized growth, Cdc42 controls membrane trafficking and the cell wall synthesis machinery through its effectors. Cdc42 activates two membrane trafficking pathways (Bendezu and Martin 2011). First it activates the formin For3 which nucleates the actin cables that will be used as tracks by the myosin-V Myo52 to deliver vesicles at the cell tips (Martin, Rincon et al. 2007). Second Cdc42 and PIP_2 recruit the exocyst, a protein complex that tethers secretory vesicles for fusion at the plasma membrane (Bendezu and Martin 2011; Estravis, Rincon et al. 2011). The protein Pob1, which binds active Cdc42 is required for both pathways (Rincon, Ye et al. 2009; Estravis, Rincon et al. 2011; Nakano, Toya et al. 2011). These two pathways are further intertwined since some of the exocyst components are carried to the cell tips via the actin cables. Cdc42 also activates the kinase Orb2, which seems to have a role in the oscillatory behavior of Cdc42 (Marcus, Polverino et al. 1995; Das, Drake et al. 2012).

Microtubule-dependent control of cell polarity and NETO

How are the growth zones defined and restricted to cell tips? The microtubule cytoskeleton plays an important role in this process. As mentioned earlier, interphase microtubule bundles run parallel to the long axis of the cell with their dynamic plus ends facing the cell tips (Tran, Marsh et al. 2001). With such an organization, growing microtubules frequently touch the cell tips where they deposit polarity factors, namely, the Tea1-Tea4 polarity complex. This complex is attached to microtubule + ends through the + TIP protein Mal3, equivalent to mammalian EB1, until microtubules undergo catastrophe near the cell tip .

Noteworthy, it has been shown that when cells are forced to grow bent in curved microchannels, it can force microtubule plus ends to interact with the lateral cortex. This is sufficient to redirect polarity factors to the lateral cortex rather than at cell tips and can generate ectopic growth zones on the lateral cortex (Terenna, Makushok et al. 2008). This formally demonstrates that microtubules can direct growth and also reveals positive feedback loops between cell shape and microtubule organization that reinforce each other to stabilize the normal rod shape morphology of fission yeast cells. The Tea1-Tea4 complex delivered at cell tips by microtubules associates stably with Mod5, a prenylated protein whose enrichment at the cell tips is itself dependent on Tea1. Mathematical modeling suggests a catalytic role for Mod5 in the maintenance and formation of the Tea1 network (Snaith and Sawin 2003; Bicho, Kelly et al. 2010).

At cell tips, the Tea1-Tea4 complex helps establishing diffusion gradients of the DYRK kinase Pom1 emanating from the cell tips (see the cytokinesis section for Pom1 gradient formation). Together, Tea1, Tea4 and Pom1 gradients control the localization of many polarity effectors that promote growth. These include the cell-wall-modifying enzymes Bgs1, Bgs3 and Bgs4, the actin cable assembly formin For3 and associated factor Bud6, actin patch components such as Sla2 and activated GTP-bound Cdc42 (Martin 2009).

Among these effects, a key event seems the localization of For3 at cell tips and activation by Cdc42. As mentioned earlier, active For3 can in turn polymerize the F-actin cables necessary to transport the vesicles delivering cell wall material to the tips. Accordingly, in a recent study a chimera with the cargo-binding domain of myosin-v and the motor domain of a kinesin proved to be sufficient to promote growth and maintain the rod shape in the absence of actin cables (Lo Presti and Martin 2011).

It is still unclear how Cdc42 talks to the Tea1-Tea4 complex. One possibility involves the Cdc42 GAP Rga4: in *pom1Δ* mutant, the localization of Rga4 expands toward one cell tip inactivating

Cdc42 and making *pom1Δ* cells unable to activate bipolar growth (NETO defective) (Tatebe, Nakano et al. 2008). This suggests that the microtubule cytoskeleton may regulate NETO by creating GAP free regions at the cell tips. A recent study also showed an asymmetrical stabilization of the microtubules at the new end when a DNA checkpoint was activated could block NETO (Kume, Koyano et al. 2011).

NETO is also under control of the cell cycle machinery: it has been recently shown that Polo recruitment to the SPB occurs relatively early during G2 and requires low levels of Cdc2 activity. Furthermore, Plo1 recruitment to SPBs in early G2 is necessary and sufficient to trigger NETO and licenses the cell to start mitosis later on (Grallert, Patel et al. 2013).

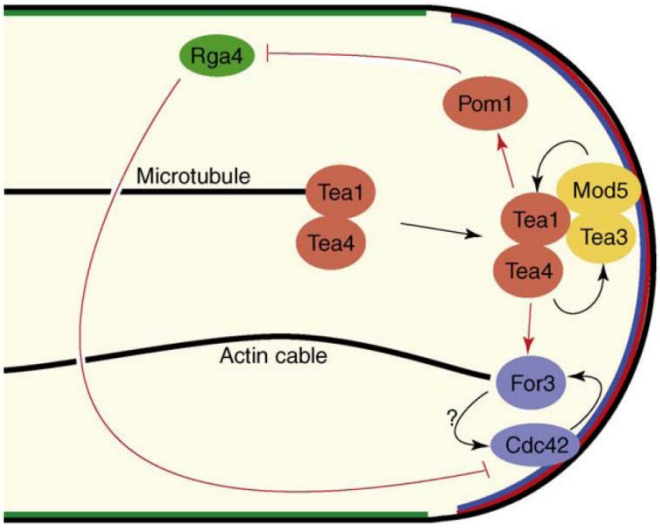


Figure 14. Model of how microtubule-associated proteins Tea1 and Tea4 initiate polarized growth at the new end for NETO. (From (Martin 2009))
The Tea1–Tea4 complex is deposited by microtubules at cell ends, where it is anchored at the cortex through direct binding to Mod5 and Tea3. At the cell tip, the Tea1–Tea4 complex recruits the kinase Pom1, which inhibits the localization of Rga4. Because Rga4 is a GTPaseactivating protein for Cdc42, this leads to activation of Cdc42 at the cell tip. Tea4 also binds directly and recruits the formin For3. Cdc42 activation and For3 recruitment lead to the assembly of polarized actin cables. Red arrows indicate theeffects of Pom1 and the Tea1–Tea4 complex. Black arrows show probable positive feedbacks that reinforce Tea1–Tea4 anchoring and actin cable assembly for polarized cell growth. For simplicity, other important NETO factors, such as Arf6, Sla2 and Bud6, are not shown.

c) Growth inactivation during mitosis

S pombe cells grow during interphase and stop growing during mitosis. Two signaling cascades play important roles in this switch, the MOR and the SIN pathways (see cytokinesis section for detailed description of the SIN). Both networks localize at the SPB and their activities are mutually exclusive. The SIN inhibits the MOR during cytokinesis through phosphorylation of the MOR component Nak1 by the SIN kinase Sid2, resulting in Orb6 kinase inactivation that blocks polarized growth as a consequence (Ray, Kume et al. 2010; Gupta, Mana-Capelli et al. 2013; Gupta, Govindaraghavan et al. 2014). The inhibition of MOR during cytokinesis is also required to avoid cell lysis due to premature cell wall digestion.

Full SIN activation is achieved by the tip protein Edt1 signaling to the SPBs as they come in proximity to cell tips during spindle elongation. Fully active SIN then inactivates Etd1 in a negative feedback loop, that eventually inactivates the SIN and resets Orb6 activity as cells exit cytokinesis (figure 15) (Garcia-Cortes and McCollum 2009).

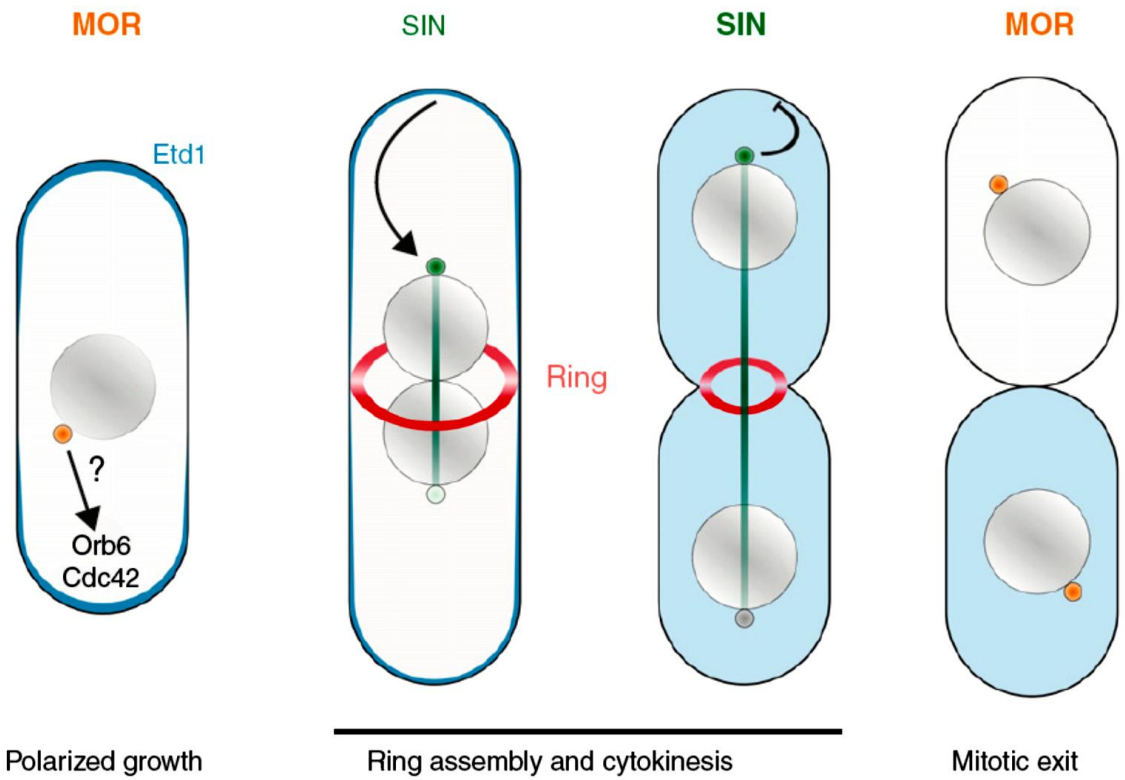


Figure 15: Antagonism between MOR and SIN pathways. (From (Hachet, Bendezu et al. 2012) Switching from polarized growth to cell division: during interphase, the MOR maintains polarized growth by acting on Cdc42 via Orb6. Etd1 is cortical and enriched at cell tips. In mitosis, SIN reaches full activation at the new spindle pole body as it comes in proximity to cortex localized Etd1 during anaphase. SIN activity in turn delocalizes Etd1 from the cortex leading to SIN inactivation at mitotic exit and the resetting of the MOR.

B. Cell division in *S. pombe*

1. Cell cycle progression and control of mitotic entry.

a) The biochemical clock of the cell cycle

Fission yeast is a well-recognized model for cell cycle studies were lots of the most important elements of the eukaryotic biochemical cell cycle clock have been discovered. In fission yeast the major player in controlling both the G1→S and the G2 → M transitions is the cyclin-dependent kinase Cdc2 kinase, known in all organisms as Cdk1. Different levels of activity of Cdc2 operate in different phases of the cell cycle. Cdk1 activity is low in G1, moderate during S-phase and G2 and high at the end of G2 and during most of M-phase.

Cdc2 associates with different cyclins during cell cycle progression. During G1, the G1-cyclin-Cdc2 takes the decision to commit into a new cell cycle. Later on, a low activity of Cyclin-B-Cdc2 triggers S-phase while a high activity is required for the G2/M transition. The Cyclin B Cdc2-complex is also known as MPF (mitosis promoting factor) as it was first discovered for its role as main mitotic inducer.

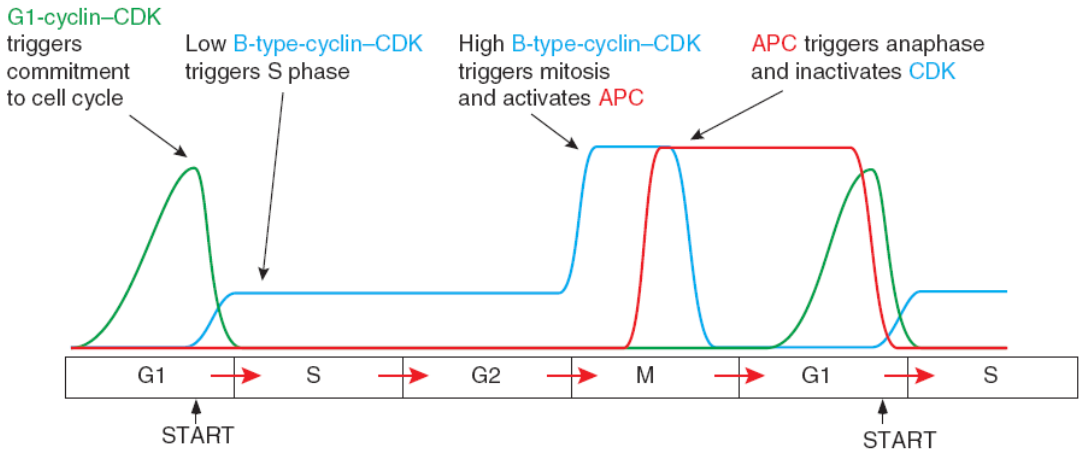


Figure 16: The major events of the cell cycle. (From (Rhind and Russell 2012)
The major events of the cell cycle are regulated by successive waves of kinase and ubiquitin ligase activity. G1-cyclin–CDK1 activity is required to initiate the cell cycle and activate B-type cyclin–CDK1 activity. Low levels of B-type-cyclin–CDK activity are sufficient to trigger S phase, but tyrosine phosphorylation by Wee1 prevents full activation, to avoid premature mitosis. Full CDK activation triggers mitosis and activates APC, which triggers anaphase and feeds back to inactivate CDK activity. Inactivation of CDK allows exit from mitosis and the reestablishment of interphase chromosome and nuclear structure in G1 phase.

There are in fact 4 different cyclins in fission yeast, the G1/S cyclins Cig1, Cig2 and Puc1 and the mitotic cyclin Cdc13 (Hagan, Hayles et al. 1988; Bueno, Richardson et al. 1991; Forsburg and Nurse 1991; Connolly and Beach 1994). While the amount of Cdc2 remains constant during the cell cycle, its activity oscillates as well as cyclin levels. Cig2 is the major G1 and S-phase cyclin. It accumulates during G1 and it disappears at the exit of S-phase. Cdc13 accumulates during G2 phase reaching its maximum concentration at the end of G2 and during mitosis.

At mitosis exit the activity of Cdc2 has to be shut down to start the next cell cycle. This is achieved in three ways. First the APC mediated ubiquitination of Cdc13 targets the mitotic cyclin for fast proteasome degradation. Secondly, the Cdc2 inhibitor Rum1 accumulates during the mitotic anaphase and persists through G1. Lastly Cdc2-Cig2 also targets Cdc13 for degradation. Rum1 is able to inhibit Cdc2-Cdc13 and Cdc2-Cig2. Activation of Cdc2-Cig2 during G1 thus requires Rum1 inactivation. This is done by the Rum1-Puc1 and Rum1-Cig1 complexes. These less abundant complexes are insensitive to Rum1, can phosphorylate it and target it to degradation by the proteasome (Benito, Martin-Castellanos et al. 1998).

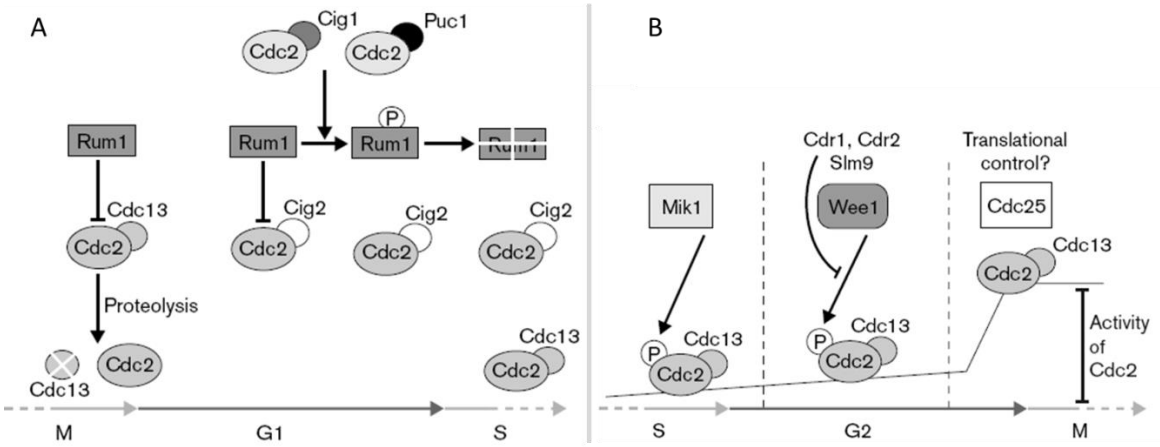


Figure 17: Models of cell-cycle-specific regulation of Cdc2. (From (Moser and Russell 2000))

(A) Cdc2 activity is regulated through its association with the four different cyclins: Cig1, Cig2, Puc1 and Cdc13. Cdc2 activity is inhibited when cells exit mitosis by degradation of its associated cyclin Cdc13. Accumulation of the Cdc2 inhibitor Rum1 ensures that Cdc2 activity is kept low throughout late M-phase and G1-phase. Late in G1-phase, phosphorylation (P) carried out by Cig1- and Puc1-associated Cdc2 targets Rum1 for degradation. In the absence of Rum1, Cdc2–Cig2 activity rises and induces entry into S-phase. Cdc13 accumulates during S-phase and it remains associated with Cdc2 until it is degraded upon exit from M-phase.

(B) Cdc2–Cdc13 activity is the major activity required for entry into mitosis. In S-phase and G2-phase, its activity is downregulated through the inhibitory phosphorylation mediated by Mik1 and Wee1. Whereas Mik1 is the main player in S-phase, its protein levels drop in G2-phase and it is left to Wee1 to inhibit Cdc2–Cdc13. Wee1 is inhibited by the protein kinases Cdr1 and Cdr2, and a protein of unknown function, Slm9. In contrast, the Cdc2 phosphatase Cdc25 accumulates during interphase, probably through translational upregulation (translational control). The counterbalance of Wee1 and Cdc25 activity changes as cell size increases and, at the proper cell size, Cdc25 is finally able to bring about Cdc2 activation, driving cells into M-phase.

b) The regulation of S-phase onset

S-phase begins with replication initiation, an event that starts simultaneously at many sites in the genome. Prior to replication initiation, a heterohexameric origin-recognition complex (ORC), which is bound to origins, recruits other proteins to these sites to form a pre-replication complex (pre-RC). Initation of replication occurs in two steps. First, the DNA is licensed for DNA replication during G1 when ORC recruits Cdc18, Cdt1 and MCM to form the pre-RC. At the beginning of S phase the actual firing of the origins occurs. Two cell cycle proteins kinases Cdc2 and Hsk1 phosphorylate the components of the pre-RC and activate DNA replication. (Leatherwood, Lopez-Girona et al. 1996; Lygerou and Nurse 1999; Ogawa, Takahashi et al. 1999).

c) The control of mitotic entry

Two major cell cycle transitions are then required to complete cell division: the G2/M transition that corresponds to mitotic entry, followed by the metaphase/anaphase transition at mitotic exit. These successive steps are triggered by the activation of Cdk1-Cdc13 complex at high levels and by the anaphase-promoting complex (APC) respectively.

i/ The balance between Wee1 and Cdc25 controls mitotic commitment

During G2 phase, Cdk1-Cdc13 activity is maintained at a low level in spite of Cdc13 accumulation and Rum1 degradation, to prevent precocious entry into mitosis. Indeed, although Cdc2 and the Cdc13 starts associating in early S-phase, the activity of the complex is inhibited by phosphorylation of Thr 14 and Tyr 15, two residues located at the ATP binding site of Cdc2. Tyr15 is the most important of the two sites and is evolutionary conserved. This phosphorylation is performed by two kinases Mik1 and Wee1. Tyr 15 phosphorylation is counteracted by Cdc25 phosphatase so that the activation of Cdk1-Cdc13 depends in the end

on the equilibrium between Mik1/Wee1 and Cdc25 activity (Russell and Nurse 1986; Russell and Nurse 1987; Den Haese, Walworth et al. 1995; Berry and Gould 1996).

This equilibrium is regulated in parts by the protein levels of Cdc25, Wee1 and Mik1 during the cell cycle. Cdc25 accumulates during G2 and disappears at mitosis (Moreno, Nurse et al. 1990). On the other hand the levels of Mik1, a protein with a short half-life, increase during S-phase (Baber-Furnari, Rhind et al. 2000) while Wee1 levels remain constant during the cell cycle. These data suggest that Mik1 has a predominant role during S-phase, while Wee1 is the main inhibitor of Cdc2-Cdc13 during G2. This may explain why *mik1Δ* cells, although short, are viable, but become inviable and exhibit catastrophic mitosis when combined with wee1-50 mutation that inactivates Wee1.

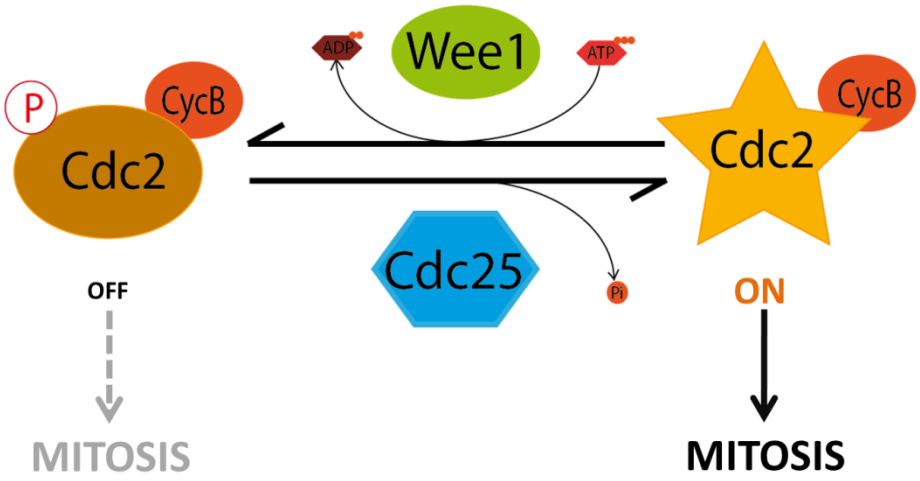


Figure 18: The basic regulation of mitosis entry

Positive and negative signaling pathways converge on Cdc2-Cdc13 to trigger the G2/M transition. Initial activation of Cdk1 may occur when Cdc13 and Cdc25 levels are high enough to overcome Wee1-dependent phosphorylation on Cdc2 Tyr15. This initial activation may then

reinforced in a positive feedback loop by the polo-like kinase Plo1 activated by Cdc2-Cdc13 , leading to Cdc25 full activation and to Wee1 inhibition.

Importantly, it has shown that a chimeric protein constituted of Cdc13 fused to Cdc2, under the control of the Cdc13 promoter, expressed in a strain where the endogenous Cdc2 and Cdc13 are inactive, is sufficient to coordinate a relatively normal cell cycle, in the absence of the G1/S cyclins, and bypasses the normal requirement for Cdc2 Y15 regulation for G2/M transition. This study shows that oscillations between low levels and high levels of Cyclin B-Cdk1 activity is sufficient to order successive S and M phase and run a minimal cell cycle clock, independently of the Wee1-dependent regulation of G2/M transition (Coudreuse and Nurse 2010). More complex systems with multiple inputs may allow the cell to better cope with the environmental stresses and to adjust to environmental changes.

ii/ Inhibition of mitotic entry by the DNA damage and replication checkpoint

DNA damage and replication checkpoint mechanism monitoring the completion of S phase can block this transition to ensure genome stability when chromosomes are broken or incompletely replicated. This involves cellular responses to DNA damage and replication-fork stalling, controlled by members of the PIK family of kinases: Tel1/ATM (ataxia telangiectasia mutated), Rad3/ATR (ATM and Rad3-related), and DNA-PK (DNA-dependent protein kinase) (Jimenez, Yucel et al. 1992; Matsuura, Naito et al. 1999; Shiloh 2003; Cimprich and Cortez 2008). These pathways block mitosis through mainly by inhibiting Cdc25 and activate in parallel the necessary DNA repair mechanisms

iii/ Control of mitotic commitment through Plo1

In addition to checkpoints, other physiological cellular inputs modulate mitotic entry by acting on the equilibrium between Cdc25 and Wee1. Mitotic entry is in particular influenced by the recruitment of Plo1 to the spindle pole body (SPB) that modulates Cdc2-Cdc13 activation feedback loop. Plo1 is recruited to the SPB by Cut12 for this function. Accordingly, an hyper-activating mutation of Cut12 (*cut12-s11*) enables Cdc25-defective cells like *cdc25-22* to enter mitosis (Hudson, Feilotter et al. 1990; Bridge, Morphew et al. 1998; Grallert, Chan et al. 2013).

A negative regulator of Plo1 recruitment to SPBs is the protein phosphatase 1 Dis2. Dis2 binds to Cut12 through a PP1 docking site (PDS), and the presence of the phosphatase inhibits Plo1 recruitment, most likely by dephosphorylation of Ser402. However, Cdc2 and the NIMA kinase Fin1 phosphorylate Cut12 during G2 to impair Dis2 binding, allowing Plo1 recruitment to the SPB (Grallert, Chan et al. 2013).

Importantly, Plo1 receives inputs from an upstream signaling pathway, the stress response pathway (SRP), itself regulated by TOR (target of rapamycin) that modulates cell size at division in response to a variety of environmental cues including stress and nutritional availability (Davie and Petersen 2012).

Accordingly, rapamycin-induced inhibition of TOR signaling advances mitotic onset, mimicking the reduction in cell size at division seen after nitrogen deprivation. On the opposite, upon TOR activation, Gcn2, a kinase which affects transcriptions levels of Pyp2 (Nemoto, Udagawa et al. 2010) gets inactivated. Pyp2 levels increase as a consequence. The phosphatase Pyp2 inhibits the MAPK Sty1. This delays mitosis onset by inhibiting Plo1 recruitment to SPBs (Petersen and Nurse 2007; Petersen 2009) that it otherwise favored by Sty1 that phosphorylates Plo1 Ser 402 to promote Plo1 association with Cut12 (Petersen and Hagan 2005; Petersen and Nurse 2007; Grallert, Chan et al. 2013).

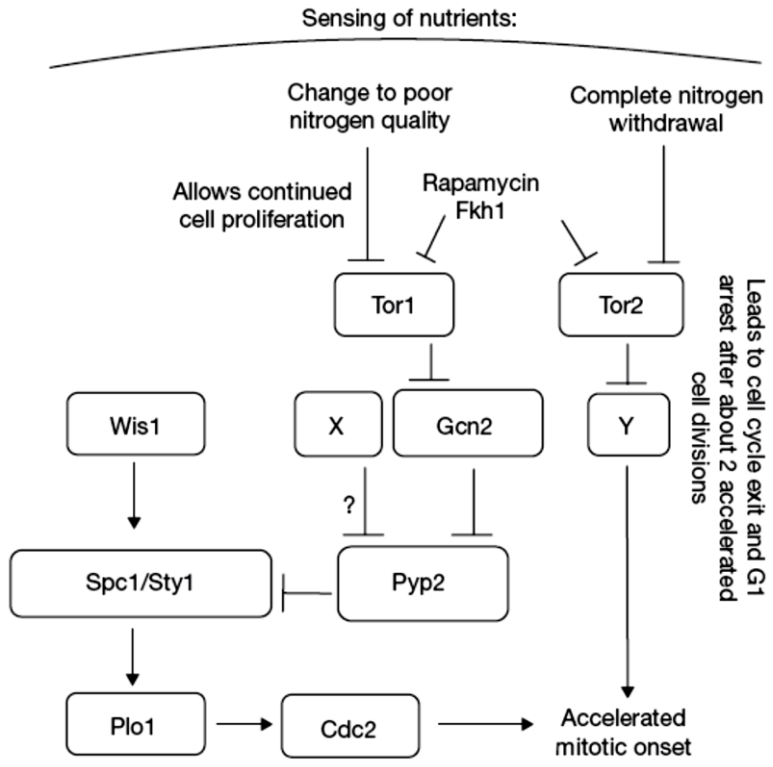


Figure 19: Proposed signalling pathways modulating mitotic commitment. (From (Petersen and Nurse 2007)

A diagram showing the proposed signalling pathways that control nutritional modulation of the cell-size control at mitotic onset and cell division. Arrows and lines indicate positive and negative signals, respectively, rather than direct interactions. X represents potential further nutrient-induced Pyp2 regulation; Y represents unknown molecules inhibited by Tor2 to block sexual differentiation

iv/ Modulation of mitotic commitment through Wee1

Regulation of Wee1 by medial cortical nodes organized by Cdr2

Wee1 needs to be inhibited for mitotic commitment. A number of cell signaling pathways converge on Wee1 to promote mitotic entry. The most important inhibitors of Wee1 discovered so far in *S. pombe* are the Change Division Response kinases Cdr2 and Cdr1/Nim1 (Russell and Nurse 1987; Young and Fantes 1987). These two kinases are similar to the Sad/Brsk kinase in mammals and Nim/Septin kinases in S. cerevisiae and belong to the superfamily of AMPKs. We will explain the importance of these relations later on, on this manuscript. *S.pombe* cells become shorter when switched to a medium with a poor nitrogen source. Cdr1 and Cdr2 were discovered during the initial genetic screenings done in fission yeast and described as unable to respond to the nitrogen shift. Indeed *cdr1Δ* and *cdr2Δ* cells remain long in poor nitrogen medium. But even in rich medium *cdr1Δ* and *cdr2Δ* cells divide at a long cell size indicating that they participate in mitotic commitment (Young and Fantes 1987; Belenguer, Pelloquin et al. 1995).

Both Cdr2 and Cdr1 were reported to directly phosphorylate Wee1. Cdr1 was also shown to inhibit Wee1 activity *in vitro* (Coleman, Tang et al. 1993; Parker, Walter et al. 1993; Wu and Russell 1993; Kanoh and Russell 1998). Genetic evidence based on cell length measurements place Cdr1 downstream of Cdr2, although each kinase receives its own regulatory inputs as we will explain later (Breeding, Hudson et al. 1998; Kanoh and Russell 1998; Morrell, Nichols et al. 2004). Some of these inputs may link cell cycle progression to cell size to control cell size at division.

Surprisingly, Cdr1 and Cdr2 localize during interphase on the middle cortex of the cell in structures that have been called medial cortical nodes. The interphase medial cortical nodes list of known components includes 8 proteins: Cdr1, Cdr2, Wee1, Mid1, Blt1, Gef2, Nod1 and Klp8. Cdr2 is the key organizer of the medial cortical nodes since the rest of node components

depend on it for a proper node localization. But the deletion of any other node component does not affect Cdr2 localization. Cdr2 has a C-terminal KA1 domain (Morrell, Nichols et al. 2004; Moravcevic, Mendrola et al. 2010; Rincon, Bhatia et al. 2014) that anchors it to the membrane and an N-terminal kinase domain separated by a long spacer. We will discuss the structural features off Cdr2 later on.

Regulation of Cdr2 nodes by Pom1 gradient

As mentioned earlier in the manuscript, Pom1 is a DYRK kinase that forms a diffusion gradient emanating from the cell tips, with decreasing concentrations towards the cell middle. Besides its function in polarity establishment, Pom1 also modulates negatively the cortical distribution of medial cortical nodes, restricting them to the cell middle as well as the activity of Cdr2 nodes towards Wee1 (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009; Bhatia, Hachet et al. 2014; Deng, Baldissard et al. 2014; Rincon, Bhatia et al. 2014).

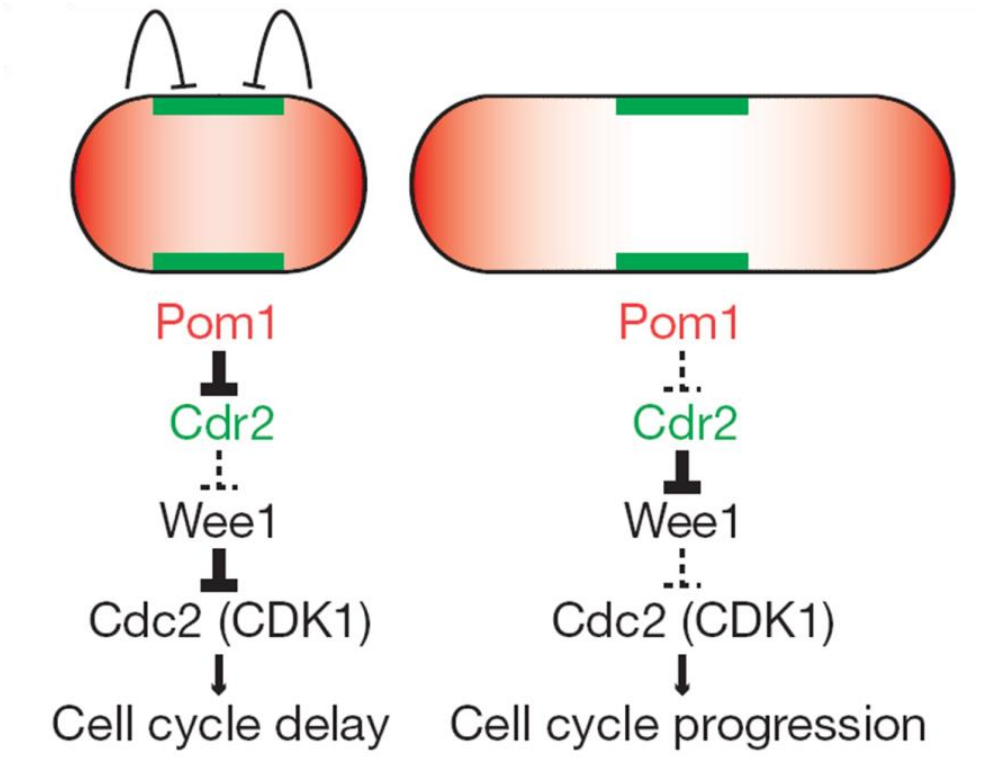


Figure 20: Pom1 overlaps with Cdr2 and prevents mitosis in small cells. (From (Martin and Berthelot-Grosjean 2009))

Model for how fission yeast cells monitor their length to control entry into mitosis. A gradient of Pom1 from cell ends overlaps significantly with Cdr2 at the middle of small cells, leading to Cdr2 inhibition and cell cycle delay. Once the cells reach a longer size, the concentration of Pom1 at the cell middle is no longer sufficient for significant Cdr2 inhibition, allowing cell cycle progression.

- Pom1 gradient establishment

As mentioned above in the morphogenesis section, the microtubule cytoskeleton delivers some polarity clues, the Tea1-Tea4 complex, to the cell tips. Pom1 is indirectly influenced by the Tea1-Tea4 complex that associates at cell tips with the phosphatase Dis2 through interactions with Tea4 (Kokkoris, Gallo Castro et al. 2014). This phosphatase favors Pom1 association with the plasma membrane by counteracting Pom1 autophosphorylation on its basic lipid-binding domain that normally results in membrane dissociation. In this way, Pom1 can efficiently associate with the membrane at cell tips where Dis2 is concentrated but dissociates gradually form the membrane as it diffuses away from the cell tips, generating the Pom1 gradient (Hachet, Berthelot-Grosjean et al. 2011).

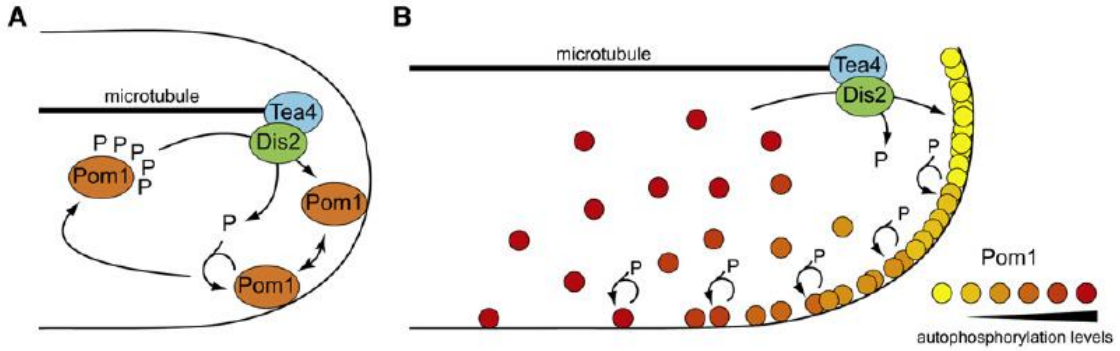


Figure 21. Model for the Formation of Cortical Pom1 Gradients. (From (Hachet, Berthelot-Grosjean et al. 2011)
(A) Local dephosphorylation of Pom1, mediated by the Tea4-Dis2 PP1 pair, which is localized to cell tips through microtubule transport, permits association of Pom1 with the plasma membrane at cell tips. Pom1 then diffuses in the plane of the membrane. Autophosphorylation leads to Pom1 detachment from the membrane. (B) Multiple autophosphorylation events may serve as a timer for shaping Pom1 gradients. After dephosphorylation and plasma membrane association, multiple rounds of autophosphorylation gradually increase the probability of Pom1 detaching from the membrane. Pom1 is shown in various shades of red indicating various degrees of autophosphorylation, from dephosphorylated (yellow) to fully phosphorylated (red).

- Shape of Pom1 gradient

It has been proposed that the shape of Pom1 gradients is stabilized by buffering mechanisms that counteract its intrinsic noise. Based on mathematical modeling, the formation of Pom1 clusters could account for such a buffering mechanism (Saunders, Pan et al. 2012) and yield to stable short range gradients.

Furthermore, this short range gradients that do not seem to extend to the cell middle as originally described (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009), even when cells are very short. Accordingly, automated analysis of Pom1 gradient shape indicates that Pom1 has similar low levels in the cell middle in short or long cells although the width of the window of lowest concentration enlarges as cells become longer (Bhatia, Hachet et al. 2014).

-Pom1 inhibition of Cdr2 medial nodes

Pom1 has two actions on medial nodes organized by Cdr2: it inhibits their distribution at non growing cell tips, in parallel to growth. Accordingly, Cdr2 nodes invade the non-growing cell tip in the absence of Pom1 (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009). Pom1 also regulates negatively Cdr2 activity towards Wee1. The best evidence for this second activity of comes from cell size measurements and epistasis studies which indicate that Pom1 acts as an inhibitor of mitotic entry acting upstream of Cdr2 (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009). Cdr2 was also shown to be a substrate of Pom1 kinase. Given the differential localizations of Pom1 forming a cell tip gradient which sources move apart when cells grow in length and Cdr2 nodes in cell middle, this data led to a model in which Pom1 gradient may provide a way to monitor cell size and transmits this information to Wee1 in order to link mitotic commitment to cell size (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009). The Pom1-Cdr2 pathway has been called the Cell Geometry Network (CGN) as a consequence.

In the initial model, Pom1 concentration is high enough on the medial cortex to inhibit Cdr2 when the cells are short but as the cells grow longer Pom1 concentration at the cell middle diminishes alleviating Cdr2 inhibition, allowing Cdr2-dependent inhibition of Wee1 through Cdr1 and leading to mitotic entry (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009). New measurements of Pom1 intensity show that Pom1 gradient is of shorter range than previously appreciated and that the concentration of Pom1 at the medial cortex of the cell is always at its lowest. Thus, this model needs to be revisited (Bhatia, Hachet et al. 2014). Several possibilities have been proposed: Pom1 could provide a constant inhibition over Cdr2 independent of cell size; Pom1 could inhibit Cdr2 activity in the short overlap region between Cdr2 nodes and Pom1 gradient, which varies as cells grow in length; Pom1 could inhibit Cdr2 in the cell tip regions where Cdr2 may transiently interact with Pom1, and this inhibition may be gradually alleviated as the window of low Pom1 concentration widens with cell size (Bhatia, Hachet et al. 2014).

Finally, Cdr2 accumulates on the cortex as cells grow longer. This accumulation is promoted by growth rather than alleviation of Pom1 inhibition of nodes assembly since it is still observed in the absence of Pom1, although the number and width of distribution of Cdr2 nodes is greater in this case (Bhatia, Hachet et al. 2014; Pan, Saunders et al. 2014). This suggested a fourth model in which mitotic commitment may be triggered by Cdr2 accumulation on the cortex, independently of Pom1 regulation (Pan, Saunders et al. 2014). Nevertheless, the fact an artificial increase of Cdr2 amounts is not sufficient to trigger a strong advance in mitosis onset, unless Pom1 is deleted, and that halving Cdr2 concentration in haplo-insufficient diploids does not change cell size at division (Bhatia, Hachet et al. 2014) strongly argues against this model.

-Molecular mechanisms of Cdr2 inhibition by Pom1

Recent studies have shown how Pom1 regulates in a different manner the distribution of Cdr2 nodes and its activity towards Wee1.

-Inhibition of Cdr2 kinase activity

Using a *pom1-as* strain in which Pom1 is sensitive to an ATP-analog showed that low levels of Pom1 inhibition affect Cdr2 activity with a shorter cell size at division, without modifying Cdr2 node distribution. This indicates that the two regulations exerted by Pom1 on Cdr2 activity towards Wee1 and Cdr2 nodes distribution are largely independent from one another (Bhatia, Hachet et al. 2014).

-Pom1-dependent inhibition of Cdr2 node assembly

Cdr2 inhibition by Pom1 is mediated indirectly by the phosphorylation of Cdr2 C-terminal tail (Bhatia, Hachet et al. 2014; Deng, Baldissard et al. 2014). Indeed, Cdr2 activation requires it to be phosphorylated in the T-loop of the kinase domain on a conserved threonine, T166, by Ssp1 kinase (Deng, Baldissard et al. 2014). Phosphorylation of Cdr2 tail by Pom1 has been shown to inhibit T166 phosphorylation by Ssp1. How Cdr2 tail phosphorylation by Pom1 impacts on Ssp1-dependent activation of Cdr2 remains unknown.

- Is the CGM an active cell size sensor?

The original CGM model postulated that Pom1 is an active cell size sensor. This role was put into question in a recent study (Wood and Nurse 2013) that showed that *pom1Δ* cells are still able to correct cell size defects generated by their asymmetrical division and are therefore homeostatic. The authors propose that although Pom1 may be involved in the inhibition of Cdr2 and in setting an absolute threshold for cell size at division, it may not function as an active cell size sensor. This result seems in conflict the study of Deng and collaborators who

showed that Cdr2 activation measured by T166 phosphorylation levels increases with cell size. This property is in principal sufficient to constitute the basis of an active cell size sensor that can transmit this information to Wee1 through Cdr1 to coordinate mitotic entry with cell size.

The apparent conflict between these two studies could be explained if additional redundant homeostatic mechanisms operate in parallel to Pom1 and can correct cell size defects in its absence. A recent screen performed by Navarro et al to identify new regulators of mitotic entry may help identifying those (Navarro and Nurse 2012).

Another argument in favor of the possibility that alternative pathways can modulate cell size at division besides the CGN and Wee1 comes from the study of Coudreuse and Nurse, 2010 mentioned earlier. This study showed that cells with a minimal cell cycle clock provided by the Cdc13-Cdc2 fusion can maintain fairly constant cell size at division, even in absence of the G1/S cyclins cig1, cig2 and puc1 and of inputs on G2/M transition through Cdc2 Tyr-15 phosphorylation by Wee1 (Coudreuse and Nurse 2010).

Inputs from the SIN and Fin1 and from TOR

Sid2-Mob1 is a SIN (septum initiation network) component that acts earlier in the cell cycle as compared to the classical SIN activation (see cytokinesis section). Sid2-Mob1 promotes mitotic commitment in G2 by activating the Fin1 kinase. Fin1 is a NIMA kinase (never in mitosis) which promotes its own destruction making Fin1 activation a transient, peak-like feature of the cell cycle. Interestingly, Fin1 has been shown to act as a negative regulator of Wee1 via the CGN pathway. Thus an early activation of the SIN pathway has a positive influence on mitotic commitment through the indirect inhibition of Wee1 (Grallert, Connolly et al. 2012). This reveals a new role for the SIN pathway in mitotic commitment.

A recent study that uses Torin1, a strong and specific ATP analog inhibitor for TORC1 (Tor2), shows that, as opposed to rapamycin inhibition, Torin1 can block cell growth without cell

death blocking the cells at G1. Before the cell growth block, Torin1 promotes a rapid entry into mitosis by increasing Cdc2 activity. This increase in Cdc2 activity was shown to be mediated by Plo1 and Cdr2 that induce a drop of Wee1 protein (Atkin, Halova et al. 2014). This is reminiscent of *S. cere*visiae where Cdr2 homolog Hsl1 has been described to induce, Wee1 homolog, Swe1 degradation (King, Jin et al. 2012). This study is a first hint of a similar mechanism in fission yeast in which TOR1C would be involved.

Inputs on Cdr1

Cdr1 also receives inputs through its inhibitors Nif1 (Nim1 inhibitory factor 1) and Skb1 (Wu and Russell 1997; Gilbreth, Yang et al. 1998; Deng and Moseley 2013). Both inhibitors bind to Cdr1 UBA domain (see section on AMPKs) . Nif1 localizes at the cell tips and could inhibit Cdr1 in the same way as Pom1 does for Cdr2 (Wood and Nurse 2013). Skb1 has a more surprising localization; it forms cortical nodes on the medial cortex that differ from Cdr2 node. Assembly of these nodes requires the membrane binding protein Slf1 (Skb1 localization factor1). Although Slb1 still localizes to the cortex in the absence of Skb1, both proteins require each other to assemble into nodes. Similar to Cdr2 nodes, the number of Skb1 nodes seems to increase with cell cycle progression. The authors hypothesis is that Slf1 sequesters Skb1 at this new nodes to promote mitotic entry by suppressing Skb1-dependent inhibition of Cdr1 (Deng, Kabeche et al. 2014).

d) Mitosis exit: control of metaphase/anaphase transition

Activation of Cdk1 and entry into mitosis leads to the activation of the anaphase-promoting complex (APC), which allows mitosis progression.

The APC is a multi-subunit E3-ligase that targets a range of mitotic proteins for ubiquitin-dependent degradation. It is regulated by the binding of two subunits: Cdc20 which acts during the metaphase/anaphase transition and Cdh1 which acts at mitosis exit and into G1. Cdc20-APC targets Securin, the inhibitor of Separase and thus allows upon activation the cleavage of cohesion complexes to release sister chromatids and allow their segregation by the mitotic spindle. Another important substrate of Cdc20-APC is Cyclin-B. The degradation of the mitotic cyclin promotes fast inactivation of Cdk1 (Wasch and Engelbert 2005).

In yeast there is an intrinsic delay between the activation of Cdk1 and the activation of APC, which usually gives the cell enough time to capture kinetochores and set up a metaphase plate. If not, a checkpoint mechanism called the spindle assembly checkpoint (SAC) monitoring sister kinetochore attachment by microtubules emanating for opposite poles prevents the activation of APC until chromosomes are ready for segregation in anaphase. A key component of this checkpoint, Mad2, signals the presence of unattached kinetochores by inhibition of Cdc20-dependent activation of the APC (Burgess, Rasouli et al. 2014).

While yeast only activates the SAC in case of problems, most metazoans activate the Spindle Assembly Checkpoint constitutively at each cell division (Burgess, Rasouli et al. 2014). This is presumably due to the longer time the complex metazoans spindles need to be assembled and align chromosomes. In effect, the Spindle assembly Checkpoint has gone from being a quality check checkpoint to a being a central signaling pathway in metazoans (Rhind and Russell 2012).

2. Cytokinesis in *S. pombe.*

Cytokinesis, the final step of the cell division, is usually performed by an acto-myosin ring. This is the case in fission yeast where the composition of the contractile ring and its mode of assembly have been studied in great details during the last 20 years (Bathe and Chang 2010; Laporte, Zhao et al. 2010; Pollard and Wu 2010; Goyal, Takaine et al. 2011; Lee, Coffman et al. 2012; Rincon and Paoletti 2012).

a) Definition of the division site

In fission yeast the contractile ring is always placed in the cell middle to produce two daughter cells of equal size (Paoletti and Chang 2000; Celton-Morizur, Bordes et al. 2004; Tolic-Norrelykke, Sacconi et al. 2004; Padte, Martin et al. 2006; Almonacid, Moseley et al. 2009).

Division plane positioning relies on the anillin-like protein Mid1, as revealed by the phenotype of *mid1Δ* cells, that most often assemble, instead of real contractile rings, strands of ring components at random angles compared to the cell long axis, in any position of the cortex with the exception of the very tips of the cell. Nevertheless, functional rings are sometimes assembled, allowing survival of a small percentage of cells in the population and demonstrating that Mid1 is not an essential component of the contractile ring but dictates where it assembles (Chang, Woollard et al. 1996; Sohrmann, Fankhauser et al. 1996; Huang, Yan et al. 2008).

To place the contractile ring in the cell middle, Mid1 associates with the presumptive division site on the medial cell cortex from early G2. This cortical localization of Mid1 is controlled by the CGN described earlier, to promote an equal division of the cytoplasm, and by the nucleus at mitotic entry, to partition chromosomes equally in the two daughter cells. Since the nucleus is normally placed in the middle by microtubule pushing forces (Tran, Marsh et al. 2001). These

two mechanism normally overlap in wild type cells and lead to robust definition of the division plane in the cell middle.

The nucleus: a positive cue for division plane positioning

Evidence for a role of the nucleus in defining the division plane came from microtubule organization mutants where the nucleus is displaced from the cell middle leading to asymmetric cell division (Toda, Umesono et al. 1983; Radcliffe, Hirata et al. 1998). These results were confirmed by active displacement of the nucleus by cell micromanipulation using optical tweezers or cell centrifugation (Tolic-Norrelykke, Sacconi et al. 2005; Daga, Lee et al. 2006). Since Mid1 has an important nuclear pool in interphase and exit the nucleus at mitotic entry in a Plo1-dependent manner (Bahler and Pringle 1998), it was proposed that Mid1 may link by export the division plane to the position of the nucleus (Sohrmann, Fankhauser et al. 1996). This was formally demonstrated using mutants of Mid1 defective for nuclear localization. These mutants are able to place the contractile ring normally in the cell middle but cannot readjust the division plane to the new position of the nucleus when it is artificially displaced from the cell middle by centrifugation (Almonacid, Moseley et al. 2009)

The CGN prepositions the division plane to the cell geometrical center in G2

In parallel to the nucleus, the CGN which has already been described for its role on the regulation of mitotic entry (see the cell cycle section) also impacts on division plane positioning by regulating Mid1 distribution on the cell cortex during interphase. Accordingly, about 80% *pom1Δ* cells divide asymmetrically, with offset and non-orthogonal septa (Bahler and Pringle 1998). Mid1 localization in *pom1Δ* cells explains this phenotype: cortical Mid1 expands towards the non-growing cell tip, shifting the division plane position towards this tip. The nucleus is unable to correct this defect since the nuclear pool of Mid1 is also reduced as a consequence of Mid1 enhanced cortical binding (Celton-Morizur, Racine et al. 2006; Padte, Martin et al. 2006).

The CGN controls Mid1 cortical localization by recruitment of Mid1 to Cdr2 medial cortical nodes, themselves restricted to the medial cortex by Pom1 gradients, as exposed earlier. Mid1 association with medial cortical nodes depends on Cdr2 the key organizer of the medial cortical nodes in a Cdr2 kinase activity-dependent manner (Moseley, Mayeux et al. 2009).

Additional components of the medial cortical nodes may stabilize Mid1 association with nodes although the precise contribution of each component is difficult to assess since node components interact in a network-like manner rather than linearly which causes redundancy between interactions (See Article 1 in the Results section and Discussion).

Interestingly, several of these additional node components namely Blt1, Gef2, Nod1 and Klp8, are components of the contractile ring that remain associated with it throughout its assembly process and constriction phase. Therefore, medial cortical nodes not only predefine the position of the division plane but also constitute true precursors of the contractile ring, preassembled during interphase.

In a recent study it has been proposed that these precursors form by fusion of Cdr2/Mid1 nodes called the type I nodes with a second type of preassembled nodes organized by Blt1, and containing Nod1 and Gef2, called type II nodes. The authors propose that type 2 nodes, may be released from the division site when the contractile ring disassembles and may then travel from the new tip towards the medial cortex by lateral diffusion where they may fuse with type I nodes (Akamatsu, Berro et al. 2014). Nevertheless, the facts that long range movements of Blt1 nodes could not be recorded (Akamatsu, Berro et al. 2014) and that Blt1 remains associated with the cell tip rather than diffuse to the whole cortex in absence of Cdr2 (Moseley, Mayeux et al. 2009) suggests an alternative model where Blt1 recruitment to Cdr2 nodes may drive the disassembly of old Blt1 nodes inherited from the previous division cycle, by competition.

Finally, another important fact is that Mid1 can associate directly with the plasma membrane through a lipid-binding amphipathic helix that can establish electrostatic interactions with acidic phospholipids such as phosphatidyl serine or phospho-inositide phosphates. This helix functions redundantly with Mid1 association to medial cortical nodes to anchor Mid1 to the cortex and define the position of the division plane (Celton-Morizur, Bordes et al. 2004). This explains why Cdr2 deficient cells have limited division plane position defects compared to Mid1 deficient cells.

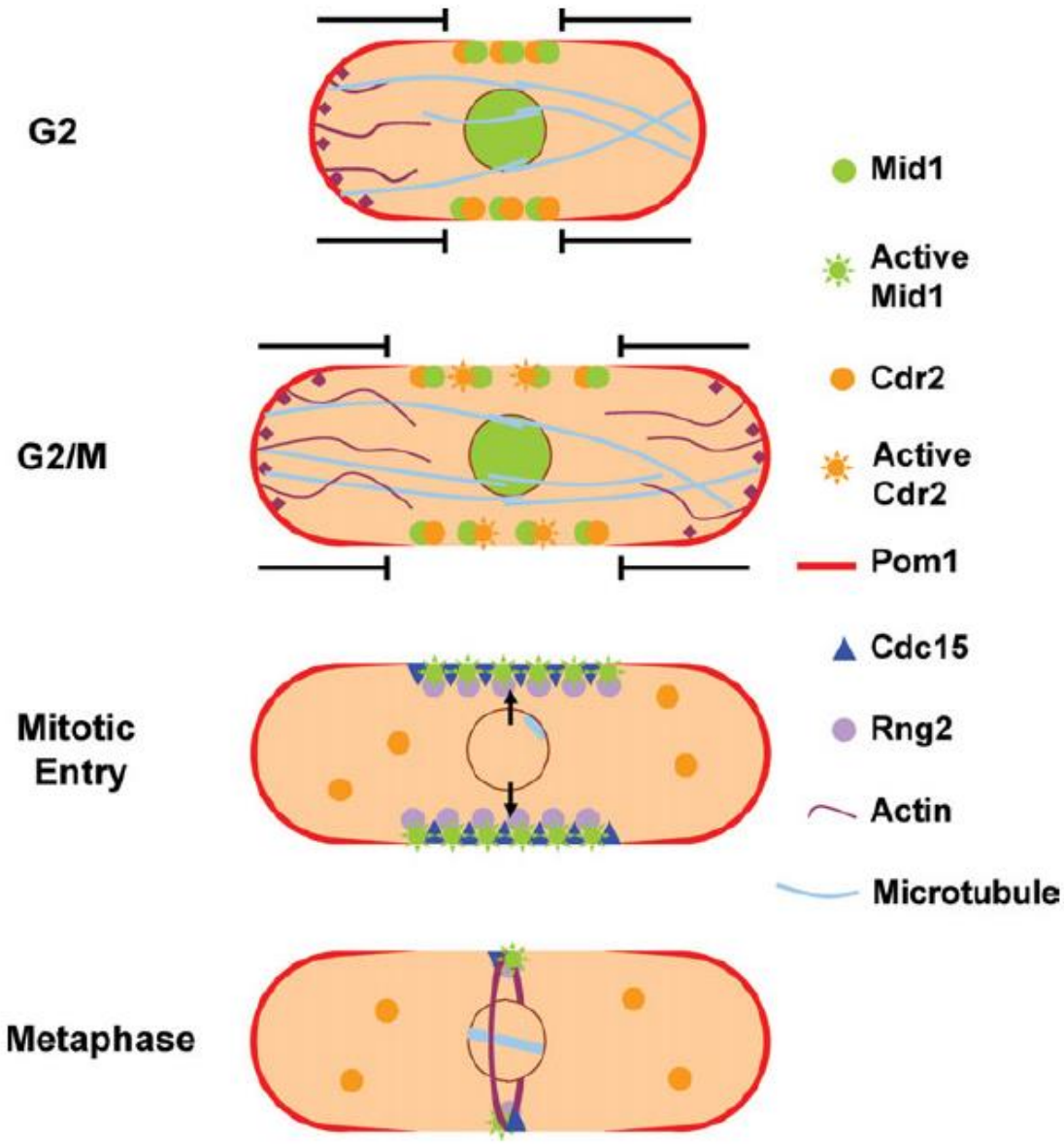


Figure 22. Major pathways regulating division plane position and mitotic entry. (From (Rincon and Paoletti 2012)

During interphase, negative signals emanating from the cell tips (Pom1 gradient and cell growth) exclude medial cortical nodes containing Cdr2 and Mid1 from the cell tips, predefining the position of the division plane in the cell middle. When cells grow in length, Pom1 inhibition is gradually relieved in the cell middle, allowing Cdr2 activation and mitotic entry. At this stage, positive signaling from the nucleus, mediated by Plo1- dependent nuclear export of Mid1, couples the position of the contractile ring to nuclear position. Concomitantly, Mid1 activation by Plo1 initiates the recruitment of contractile ring components such as Rng2 or Cdc15 to medial cortical nodes, leading to the assembly of a medially-placed contractile ring upon compaction of the cortical nodes. This double signaling mechanism favors an equal division of the cytoplasm as well as proper segregation of the chromosomes in the two daughter cells. Cdc15 localization at the cell tips during interphase is not represented.

b) Assembly of the contractile ring

Contractile ring assembly starts immediately at mitosis onset by maturation of medial cortical nodes. This maturation process corresponds to the sequential recruitment of several contractile ring components essential for its assembly. This includes the IQGAP Rng2 (Eng, Naqvi et al. 1998), myosin II and its regulatory chains Rlc1 and Cdc4 (Streiblova, Hasek et al. 1984; Bezanilla, Wilson et al. 2000; Le Goff, Motegi et al. 2000; Motegi, Nakano et al. 2000; Naqvi, Wong et al. 2000), the F-Bar protein Cdc15 (Fankhauser, Reymond et al. 1995), the formin Cdc12 (Chang, Drubin et al. 1997) responsible for the nucleation of actin cables.

Mid1 is a critical factor triggering this process, but needs to be activated by the Plo1 kinase to initiate the recruitment and scaffolding of essential ring components (Wu, Kuhn et al. 2003; Celton-Morizur, Bordes et al. 2004; Motegi, Mishra et al. 2004; Coffman, Nile et al. 2009; Saha and Pollard 2012). Indeed, Plo1 triggers both Mid1 massive export from the nucleus, but also activates Mid1 scaffolding function for contractile ring components by phosphorylation of several sites in Mid1 N-terminus (Almonacid, Celton-Morizur et al. 2011). These phosphorylations allow the binding of the IQGAP protein Rng2 required in turn the recruitment of myosin II (Almonacid, Celton-Morizur et al. 2011; Padmanabhan, Bakka et al. 2011).

Time lapse imaging of these ring components using the separation of SPBs as a temporal reference has allowed to determine the order of recruitment of these components at medial cortical nodes (Wu, Kuhn et al. 2003). Quantitative analysis further revealed the amount of each protein per cytokinetic node (Wu, Sirotkin et al. 2006). Each node contains 20 molecules of Mid1 and recruits 20 molecules of Rng2 and 20 dimers of myosin II (heavy chain Myo2 and light chains Cdc4 and Rlc1) 10 minutes before the separation of the SPBs. Ten minutes later, 20 molecules of the F-BAR protein Cdc15 and two dimers of the formin Cdc12 join the nodes (Wu and Pollard 2005; Wu, Sirotkin et al. 2006). The recruitment of all this components is an actin

independent process (Wu, Kuhn et al. 2003; Wu, Sirotkin et al. 2006; Takaine, Numata et al. 2014).

The interactions between the different cytokinetic node components were difficult to establish. Cdc4 interacts directly with Rng2 and with myosin II heavy chain Myo2 (Eng, Naqvi et al. 1998). An interaction between the tail of Myo2 and Mid1 was proposed to be regulated by the dephosphorylation Myo2 Ser1444 based on the behavior and lethality of the phospho-mimetic Myo2 mutant (Motegi, Mishra et al. 2004). However, another study showed that this mutant is in fact functional, and the lethality observed in the first study was caused by overexpression (Sladewski, Previs et al. 2009).

The recruitment of Cdc12 and Cdc15 at the medial cortex is interdependent (Chang, Drubin et al. 1997; Chang 1999) and these two proteins interact with each other through their N-terminal ends (Carnahan and Gould 2003).

Based on these initial data and on additional studies testing systematically the relationship between the cytokinesis node components (Laporte, Coffman et al. 2011), have eventually proposed that Mid1 organizes two independent modules. The first module includes Rng2 and the myosin II light chains Cdc4 which cooperate to recruit myosin II heavy chain Myo2 and the regulatory myosin II chain Rlc1. The second module is represented by the F-BAR protein Cdc15. Both modules contribute to the recruitment of the formin Cdc12 (figure 23).

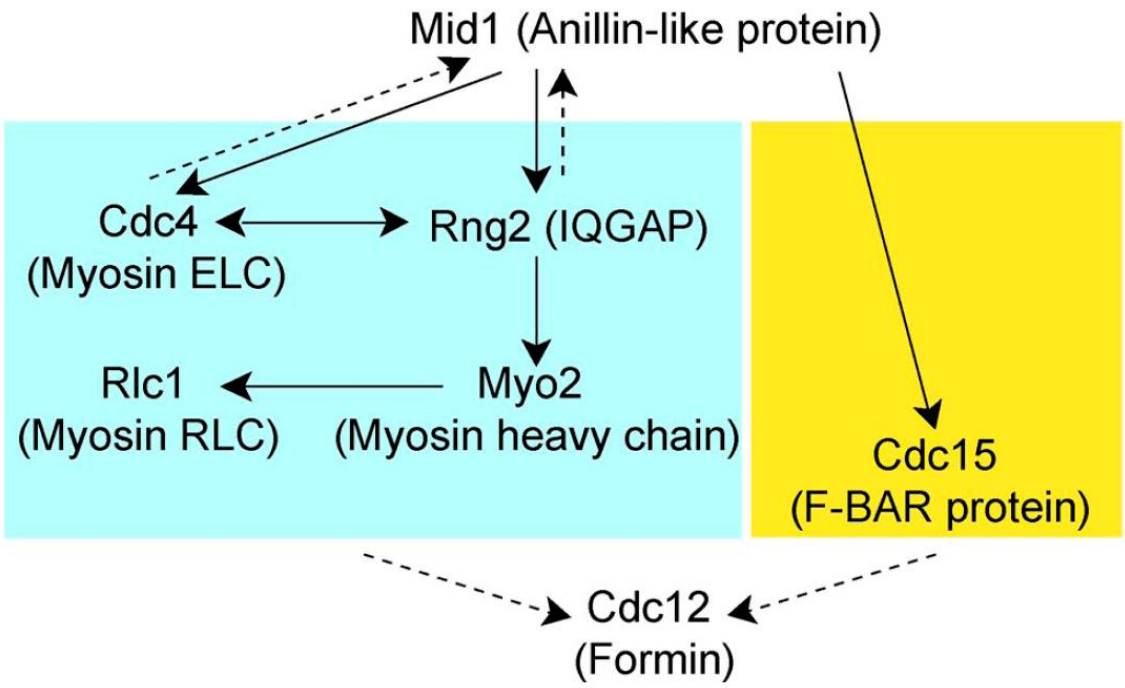


Figure 23: Genetic dependencies for node localization of Cdc15 and Cdc12 and node assembly pathways. (From (Laporte, Coffman et al. 2011))
The localization hierarchy for cytokinesis node assembly. The complete and partial dependencies of node localization on a specific protein are depicted by solid and dashed lines, respectively. The two modules are colored differently

Origin and assembly of the contractile ring F-actin

The formin Cdc12 (Chang, Drubin et al. 1997) is the major provider of F-actin filaments for the contractile ring. F-actin assembly by Cdc12 also requires the profilin Cdc3 (Kovar, Kuhn et al. 2003)(see also the cytoskeleton section). F-actin assembly was reported to start 2 minutes after SPB separation soon after the recruitment of Cdr12 to medial cortical nodes (Lu and Pollard 2001; Vavylonis, Wu et al. 2008; Coffman, Nile et al. 2009). Cdc12 anchors the actin filaments to the cytokinetic cortical nodes by their barbed end while pointed ends extend outward. Time-lapse imaging using GFP-CHD (Calponin Homology Domain) to stain F-actin in living cells confirmed this point (Vavylonis, Wu et al. 2008; Coffman, Nile et al. 2009).

In contrast to For3, Cdc12 does not have an auto-inhibitory mechanism (Yonetani, Lustig et al. 2008). The tropomyosin Cdc8 binds growing actin filaments and modulates their dynamics: in vitro it can increase the elongation speed but it can also block Cdc12 (Skau, Neidt et al. 2009). This mechanism has been proposed to work as the regulator of the length of actin filaments in the absence of an autoinhibition mechanism on Cdc12.

Recently, it was proposed that in addition to the pool of F-actin polymerized by Cdc12 in early mitosis, some F-actin originates from preassembled F-actin cables that were shown to flow towards the medial cortex in early mitosis (Huang, Huang et al. 2012). Nevertheless, this pathway may not be sufficient for ring assembly (Coffman, Sees et al. 2013). In sharp contrast, a fragment of Cdc12 was shown to be sufficient to trigger ring assembly, bypassing the normal signaling pathways that normally induce ring assembly (Yonetani and Chang 2010). Finally, the formin For3 is also present at the division site where it is recruited by Cdc15. It may also cooperate with Cdc12 to produce F-actin *de novo* during cytokinesis (Coffman, Sees et al. 2013).

In addition, the IQGAP rng2 may contribute to the contractile ring actin network. Rng2 was shown to be involved in the generation of contractile ring F-actin and simultaneously bundle the filaments and regulates their dynamics by counteracting the effects of Adf1, thus enabling the reconstruction of F-actin bundles (Takaine, Numata et al. 2009).

Compaction of mature nodes into a contractile ring

- The Search, Capture, Pull and Release model

The compaction of the band of medial cortical nodes into a contractile ring starts when Cdc12 arrives to the nodes and polymerization of actin filaments starts. Nodes then move towards each other during 10 minutes until they form a ring (Vavylonis, Wu et al. 2008). *In vivo* observations of F-actin and myosin II coupled to mathematical modeling suggested a

mechanism for the coalescence of the nodes into a ring: node coalescence may depend on traction forces exerted by Myosin II present in one node on actin filaments polymerized from another node. One important parameter for the success of the simulations using this model is the existence of short-term interactions between neighboring nodes, which also fits with in vivo observations of F-actin. This model of ring compaction has been called the SCPR (Search, Capture, Pull and Release).

One main criticism made to the SCPR model is that it does not account for ring assembly in the absence of precursor nodes (Hachet and Simanis 2008; Huang, Yan et al. 2008). The existence of a Mid1 independent pathway controlled by the SIN and Cdc15 may be responsible for this, independently of the SPCR.

Myo2 activity is essential to the SCPR model. The UCS protein Rng3 is necessary for Myo2 activity (Lord and Pollard 2004). Even if Rng3 was not detected in the nodes sofar, possibly due to weak amounts, Rng3 mutants show a slow down of node compaction suggesting that Rng3 indeed controls the activation of Myo2 during node compaction.

Recent data further show that the F-actin cross-linkers α-actinin Ain1 and fimbrin Fim1 significantly contribute to the compaction of the ring by aligning F-actin filaments (Ojkic, Wu et al. 2011; Laporte, Ojkic et al. 2012).

-The aster model

An alternative model for ring assembly was proposed: the aster or spot/leading cable model. This model was originally established from observations of “asters” of F-actin at mitosis entry after cell fixation and staining with rhodamine-phalloidin (Arai and Mabuchi 2002). Medial spots of Cdc15 and Cdc12 were also observed (Chang 1999; Carnahan and Gould 2003) which were interpreted as initiation points for the nucleation of F-actin. Electron microscopy studies

finally showed that early rings contain antiparallel actin filaments that could emanate from a single spot (Kamasaki, Osumi et al. 2007).

In this model, the Cdc12 spot moves to the medial cortical zone and nucleates linear actin filaments from a single point to form an aster. A leading cable grows from this aster and encircles the cell to form the initial contractile ring (Chang 1999; Arai and Mabuchi 2002; Carnahan and Gould 2003).

Nevertheless, labeling the actin with GFP-CHD or Life-act did not allow to see an actin aster, the observation of the Cdc12/Cdc15 spot is not always reproducible and might possibly result from protein tagging and finally, the spot progenitor spot, essential for the spot/leading cable model, usually disappeared without nucleating actin filaments (Vavylonis, Wu et al. 2008; Coffman, Nile et al. 2009). This puts the aster model into question for wild type cells. This model may however account for SIN induced ring assembly in absence of medial corticla nodes (Hachet and Simanis 2008).

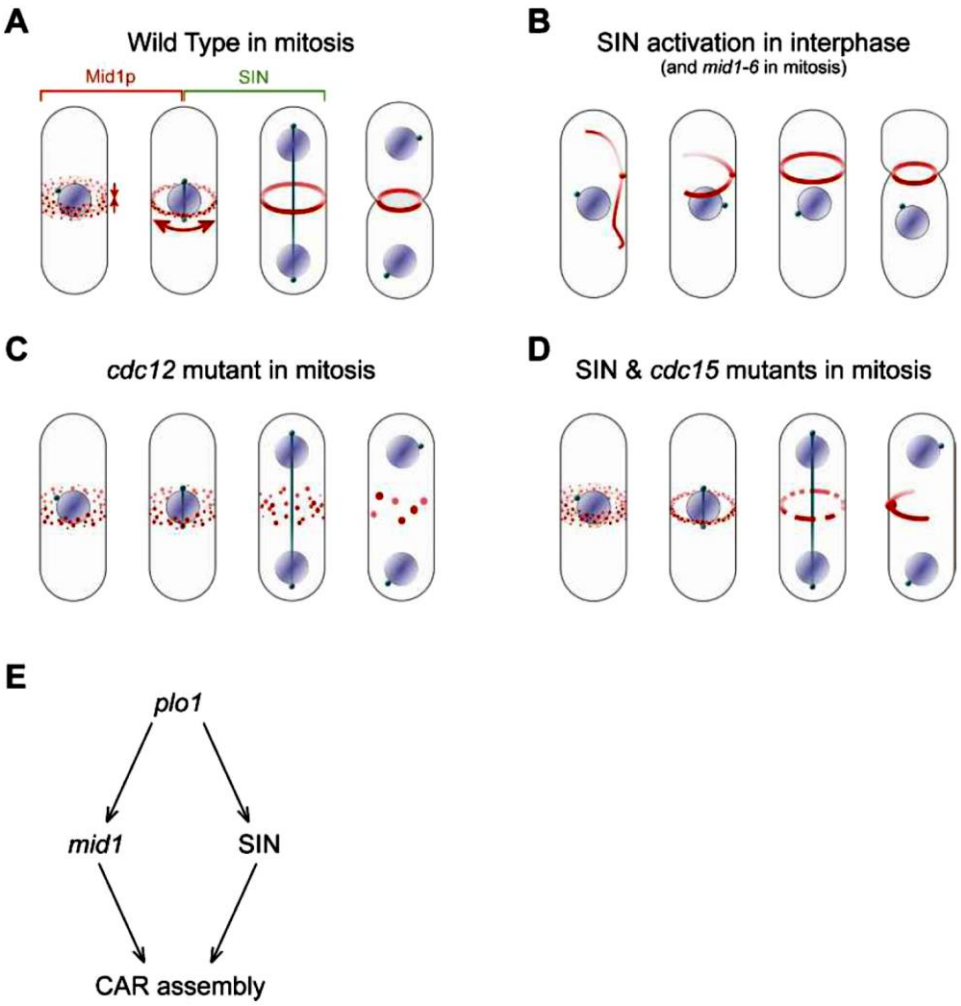


Figure 24. Models for contractile ring assembly. (From (Hachet and Simanis 2008))
Models recapitulating the three phases of contractile ring assembly in situations described in this study. (A) In wild-type mitosis, ring assembly is initiated by the formation of a cortical network of ring components that undergoes lateral condensation, giving rise to a nonhomogeneous ring precursor. This structure eventually matures by recruitment of additional factors such as Cdc15p, displaying the uniform distribution of ring components distribution that characterizes a functional ring. (B) When the SIN is activated in interphase, ring assembly is initiated by the formation of a Mid1p-independent filamentous actomyosin structure that eventually forms a ring structure. This structure is then competent for contraction, although it contracts more slowly. This mode of assembly is also observed in mid1 mutants in mitosis. (C) In a cdc12 mutant, ring assembly is initiated correctly in that it allows for the formation of a cortical network; however, this network fails to coalesce by lateral condensation and instead remains loose. (D) In SIN and cdc15 mutants, ring assembly proceeds correctly until lateral condensation of the cortical network. Thereafter, the ring precursor fails to mature, remains nonuniform, showing gaps and clumps of ring components, and, in the case of SIN mutants, fails to recruit Cdc15p. Finally, the defective rings seem to collapse, perhaps by a process involving contraction. (E) Model for the coordination of CAR assembly by plo1 controlling the two key regulators mid1 and the SIN. The SIN and Mid1p are both important for assembly of a functional CAR and cooperate to effect this. Regulation of both by Plo1p provides an attractive means of orchestrating CAR assembly during mitosis.

Ring assembly without medial cortical nodes

As mentioned earlier, functional contractile rings can assemble in absence of cortical node precursors indicating that an alternative pathway for ring assembly exists (Huang, Yan et al. 2008). The SIN network (see the SIN paragraph below for details) controls this second pathway (Hachet and Simanis 2008). Accordingly, compromising Mid1 and SIN functions at the same time prevents ring assembly altogether and is lethal.

The SIN may regulate several ring components by controlling their phosphorylation state. One target is the F-Bar protein Cd15 (Hachet and Simanis 2008), which may be dephosphorylated by Clp1, a downstream effector of the SIN pathway (see SIN paragraph below) that normally associates with Mid1 at the division site (Clifford, Wolfe et al. 2008) and counteracts Cdc2-dependent phosphorylations (Cueille, Salimova et al. 2001; Trautmann, Wolfe et al. 2001). The dephosphorylation of Cdc15 by Clp1 creates a conformational change from a closed to an open conformation that promotes Cdc15 oligomerisation, scaffolding activity and membrane anchoring. Accordingly, a mutant of Cdc15 with reduced phosphorylation was shown to appear precociously at the division site and induces the assembly of filamentous structures containing most contractile ring components. This indicates that Cdc15 has a key scaffolding role for ring assembly which is temporally regulated by its phosphorylation state (Roberts-Galbraith, Ohi et al. 2010).

A recent study identified Cdc12 as a second important target of the SIN for contractile ring assembly in absence of precursor nodes. Cdc12 is the substrate of the SIN kinase Sid2. Sid2 phosphorylates a C-terminal oligomerization domain of Cdc12 that also confers F-actin bundling activity. This prevents Cdc12 clustering and promotes contractile ring assembly when mid1 function is compromised (Bohnert, Grzegorzewska et al. 2013).

Maturation of the ring

Aftere ring compaction, during aprroximatively 25minutes, the ring keeps a constant diameter. During this time, additional proteins required for ring constriction are recruited such as the non conventional myosin Myp2, or the capping proteins Acp1 and Acp2.

Septins and Mid2, a second fission yeast anillin-like protein that regulates septin dynamics accumulate close to the ring during this period, but then form two rings that define the bounderies of the furrow during the ring contraction period (Berlin, Paoletti et al. 2003; Tasto, Morrell et al. 2003; An, Morrell et al. 2004). The recruitment of septins to the contractile ring requires the presence of actin filaments (Wu, Kuhn et al. 2003).

Another set of proteins that join the ring during maturation are the F-BAR proteins Imp2, the C2-domain protein Fic1 and the paxilli Pxl1. These proteins keep the integrity of the ring during contraction (Wachtler, Huang et al. 2006; Ge and Balasubramanian 2008; Roberts-Galbraith, Chen et al. 2009). Imp2 and Cdc15 keep the ring anchored to the membrane through their BAR domains while they interact with Fic1 and Pxl1 through their SH3 domains. Noteworthyly, interactions with Fic1 and Pxl1 is promoted by the SIN-induced dephosphorylation of Cdc15 described earlier (Roberts-Galbraith, Ohi et al. 2010).

During this period eventhough there is no contraction, a fast turnover of ring components was observed by FRAP. The phosphatase Clp1 which is loaded into the ring by Mid1 seems to be play a role in this process as a stabiliizer (Clifford, Wolfe et al. 2008). Mid1 leaves the ring at the end of this ring-maturation period (Sohrmann, Fankhauser et al. 1996) but the physiological role of this dissociation is not established yet.

c) Ring constriction and septum assembly

The ring starts contracting at the end of anaphase, 35 minutes after SPB separation. This step takes around 30 minutes. Ring constriction is tightly coupled to the synthesis of the septum of cell wall material by glucan synthases.

Mechanism of ring constriction

Ring contraction may depend on myosin II pulling on the actin filaments. The absolute amount of myosin II remains constant as contraction proceeds, but Rng2, Cdc12 and actin filaments diminish (Wu and Pollard 2005). F-actin thus needs to depolymerize as constriction proceeds in order to avoid a massive thickening of the ring as it contracts. This hypothesis has been confirmed in electron microscopy studies (Kamasaki, Osumi et al. 2007).

Recent *in vitro* studies in semi-permeabilized cells confirm that the ring contracts in an ATP- and myosin-II-dependent manner but surprisingly actin polymerization or disassembly is not required for contraction, while the addition of actin-crosslinkers can block it (Mishra, Kashiwazaki et al. 2013).

An important fact to consider for ring constriction is that fission yeast cells have a high turgor pressure that must be counteracted to allow furrow ingression. It has been proposed that the force generated by the contractile ring may not be sufficient to ingress the furrow. This force may rather be provided by the assembly of the septum, under the control of the contractile ring. Accordingly, contractile ring disassembly once the septum has started polymerizing does not prevent furrow ingression (Proctor, Minc et al. 2012).

Myosin II light chain Rlc1 is phosphorylated by Orb2 kinase on Ser35 and Ser36. These phosphorylations were proposed to avoid cut phenotypes and preserve the genomic integrity. (Loo and Balasubramanian 2008). Phosphorylation of these sites were also shown to accelerate ring constriction. The timing of ring contraction is also controlled in part by Myo2

phosphorylation on Ser 1444, shown to promote the initiation of contractile ring constriction (Sladewski, Previs et al. 2009).

Temporal control of septation by the SIN pathway

Aside myosin II phosphorylation, the major temporal control of ring constriction and septum assembly is ensured by the SIN pathway (Septation Initiation Network) that couples it to mitotic exit.

The SIN is a pathway homologous to the MEN in *S. cerevisiaae* (Mitotic exit network) necessary for Cdk1 inactivation (McCollum and Gould 2001). The SIN localizes at the SPB and is necessary for ring contraction and septum synthesis. Classically it has been considered that the SIN was activated at the exit of mitosis, but more and more studies show evidence of activity of SIN kinases as early as mid G2, with different roles in cell cycle regulation and mitosis commitment as explained earlier in this manuscript (Grallert, Connolly et al. 2012).

There are two kinds of SIN mutants (Krapp and Simanis 2008):

-Mutants of activator elements *cdc7, cdc11, cdc14, etd1, mob1, sid1, sid2, sid4* and *spg1* which block the pathway give rise to multinucleated cells (Nurse, Thuriaux et al. 1976; Creanor and Mitchison 1990; Fankhauser and Simanis 1993; Fankhauser and Simanis 1994; Jimenez and Oballe 1994; Schmidt, Sohrmann et al. 1997; Sparks, Morphew et al. 1999; Guertin, Chang et al. 2000; Salimova, Sohrmann et al. 2000). These mutants assemble rings that will fragment afterwards due to their inability to contract.

-Mutants of negative regulators of the pathway *cdc16* and *byr4* that make several rounds of cytokinesis giving rise to multiseptated cells (Minet, Nurse et al. 1979; Fankhauser, Marks et al. 1993; Song, Mach et al. 1996).

A large amount of work has been done to characterize and order components of the SIN (Balasubramanian, Bi et al. 2004; Krapp, Gulli et al. 2004; Wolfe and Gould 2005). The proteins of the SIN pathway are sequentially recruited during the cell cycle to the SPBs and they anchor through the platform made by the scaffolds Cdc11 and Sid4. The SIN activity is controlled by the GTPase Spg1 that can switch from the inactive GDP bound form to the active GTP bound form. Spg1 activity is negatively regulated by a bipartite GAP composed of Byr4 and Cdc16. while Spg1 activation triggers a kinase cascade composed of Cdc7, the Sid1/Cdc14 complex, and the Sid2/Mob1 complex. (Roberts-Galbraith and Gould 2008).

How the SIN controls septum synthesis and ring constriction remains unclear at this stage. A recent publication suggests that Blt1 may have a role in this process: Blt1 favors the recruitment of the SIN kinase complex Sid2/Mob1 which may in turn promote the recruitment of Clp1 phosphatase and Bgs1 β-glucan synthase (Goss, Kim et al. 2014).

Importantly, a major upstream regulator of the SIN is the kinase Plo1 that associates with Sid4 (Tanaka, Petersen et al. 2001). Interestingly, Plo1 association with Sid4 is blocked by ubiquitination of Sid4 by the ubiquitin ligase Dma1 when the mitotic spindle checkpoint is engaged, in order to delay cytokinesis onset and avoid damaging the DNA (Johnson, Collier et al. 2012). Since Plo1 also activates ring assembly through Mid1 and through the SIN as discussed above, it can be considered as a master kinase ensuring the coordination of various cytokinetic events and coordinating them with cell cycle progression.

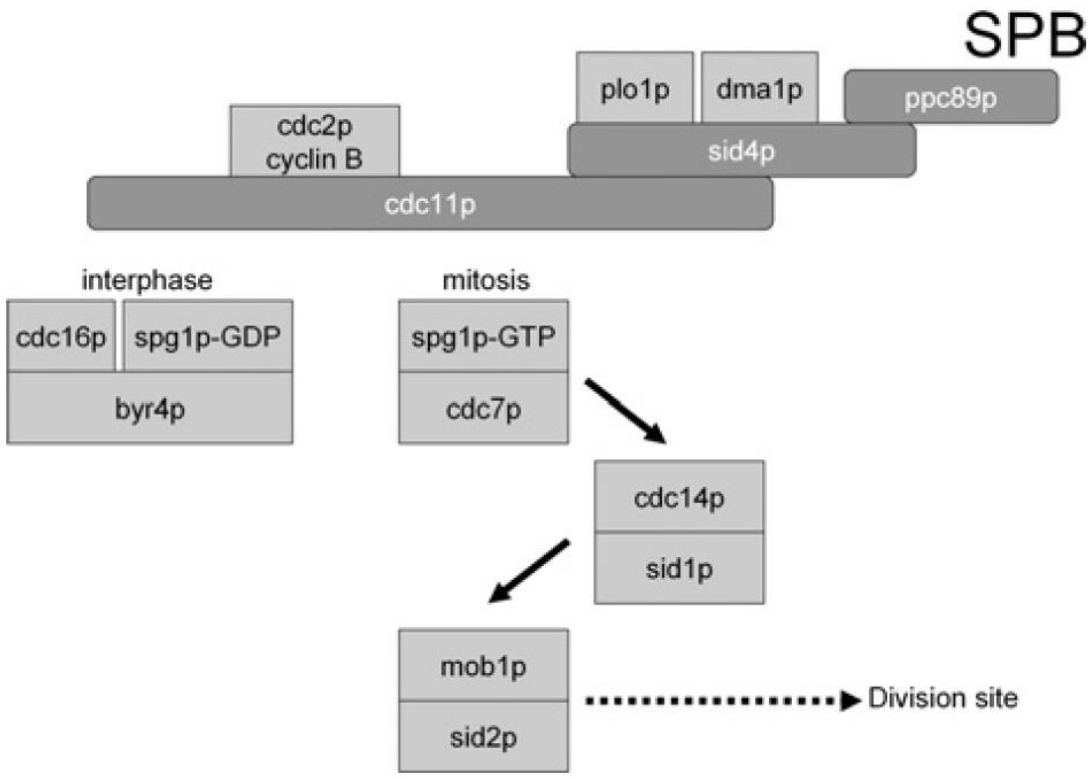


Figure 25: The main components and some regulators of the SIN (From (Krapp and Simanis 2008))
The scaffold proteins are shaded dark grey and the core components and regulators are shaded light grey. In interphase, the GTPase spg1p and its GAP associate with the SPB. In contrast, duringmitosis, the transducers of the SIN signal, cdc7p, cdc14p, sid1p,mob1p and sid2p, associate with the SPB. The arrows indicate the presumed order of action; the sid2p-mob1p protein kinase also associates with the contractile ring. Note that these proteins behave differently at the two poles of the mitotic spindle during anaphase.

d) Daughter cell separation

The septum of *S.pombe* is composed by a primary septum flanked by two secondary septa on each daughter cell sides. The deposition of the primary septum depends on the contraction of the ring (Liu, Wang et al. 1999; Liu, Tang et al. 2002) and it is done centripetally. Afterwards each daughter cell assembles septum material on their sides to build the secondary septum. The primary septum is constituted by 1,3-β-glucan while the secondary septum is made by 1,3-β-glucan 1-6 branched and Galactomannan (Humbel, Konomi et al. 2001; Sugawara, Sato et al. 2003).

The primary septum is synthetized by the sub units Cps1 and Bgs4 of the 1,3–β–glucan synthase (Le Goff, Woollard et al. 1999; Liu, Wang et al. 1999; Cortes, Ishiguro et al. 2002; Cortes, Carnero et al. 2005). This complex is regulated by the small GTPase Rho1 (Arellano, Duran et al. 1996). The physical separation of the daughter cells requires the digestion of the primary septum by the 1,3-β-glucanase Eng1 (Martin-Cuadrado, Duenas et al. 2003), but also the erosion of the mother cell walls surrounding the septum by the 1,3-α-glucanase Agn1 (Dekker et al., 2004; Garcia et al., 2005). This step finishes the process of cytokinesis.

C. The AMPK family of kinases

Most of this thesis work is focused on the kinase Cdr2 which belongs to the Calcium Calmodulin dependent kinase (CAMK) super-family, in the group of AMPK like kinases and subfamily of SAD kinases founded by *caenorabdhitis elegans* Sad-1 kinase (Synapses of Amphids Deficient 1) (Manning, Plowman et al. 2002). Cdr2 and its fission yeast homolog Cdr1 are therefore closely related to other fission yeast AMPK-like kinases including the AMPK Ucp9/Ssp2, the MARK kinase Kin1 and the CAMK like kinase Ssp1 (figure26).

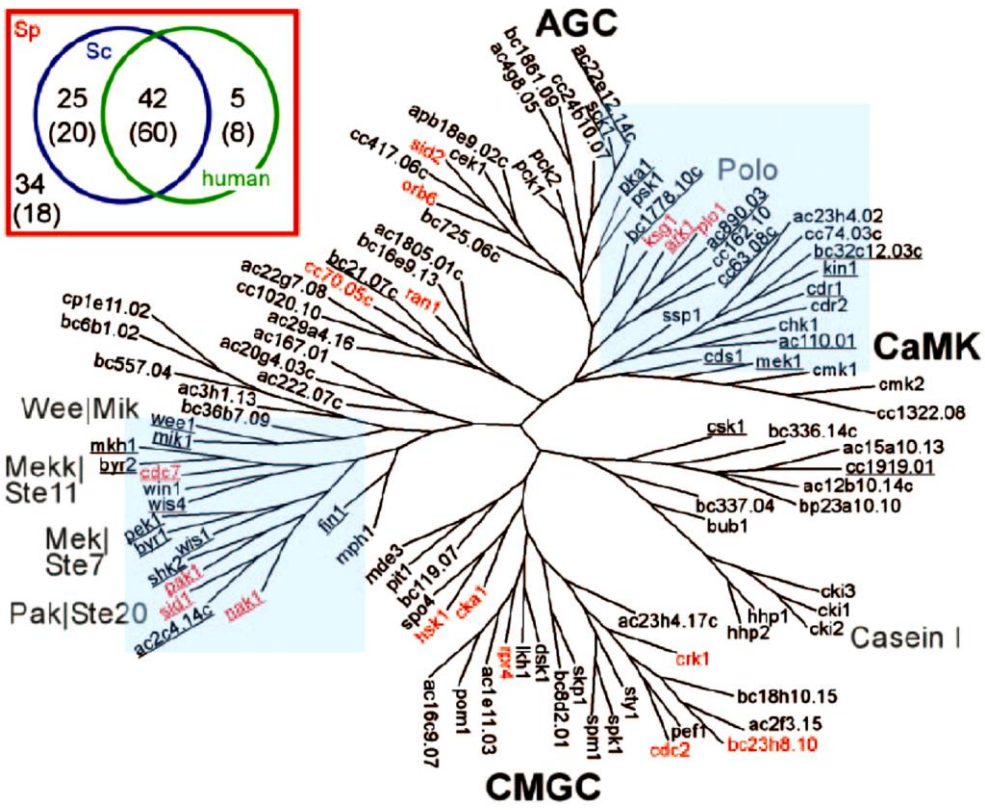


Figure26: Unrooted phylogenic tree of the 106 protein kinases in S. pombe. (From(Bimbo, Jia et al. 2005))

Seventeen essential protein kinases are marked in red, and 31 kinases containing tyrosine phosphorylation signatures are underlined. Two regions of the phylogenic tree shaded blue indicate that the region is enriched with tyrosine kinase signatures. AGC, CaMK, and CMGC indicate protein kinase groups, and Polo, Casein I, Wee/Mik, Mekk/Ste11, Mek/Ste7, and Pak/Ste20 indicate protein kinase families that do not belong to the AGC, CaMK, and CMGC groups. The inset shows a schematic representation of protein kinase orthologs in S. pombe (Sp), S. cerevisiae (Sc), and human. One hundred six eukaryotic protein kinase catalyticdomain-containing proteins were selected in S. pombe, 119 in S. cerevisiae, and 491 in human. Analysis of orthologs showed that of 106 S. pombe protein kinases, 67 (25 plus 42) have orthologs in S. cerevisiae and 47 (42 plus 5) in human. Among these, 42 appeared to have orthologs in both S. cerevisiae and human. Numbers in parentheses indicate the numbers of nearest homologs

Cdr2 and Cdr1/Nim1 orthologs in humans are the BRSK2/SAD-A and BRSK1/SAD-B kinases respectively. In *S.cerevisiae*, they correspond to the Septin kinases Kcc4 and Gin4 for Cdr2 and Hsl1 for Cdr1 (see paragraph below for a detailed description of their functions).

Mammalian true AMPKs are heterotrimeric complexes composed of an α catalytic subunit and 2 regulatory subunits, involved in energy regulation (Zhou, Myers et al. 2001). Their budding yeast ortholog is the sucrose non-fermenting 1 (Snf1)(Amodeo, Rudolph et al. 2007) equivalent to fission yeast Ucp9/Ssp2 (Matsuzawa, Fujita et al. 2012).

Humans have four MARK isoforms involved in lots of different functions from cell cycle regulation, cell polarity, neuronal migration, and cell signaling (for reviews see (Goldstein and Macara 2007; Matenia and Mandelkow 2009) while budding yeast has 2 Kin1 and Kin2 (Pallier, Valens et al. 1993) and fission yeast a single MARK Kin1 (La Carbona, Allix et al. 2004).

Other AMPK like kinases in human are NUAK1, NUAK2, QIK, QSK, SIK and MELK which functions remain poorly characterized (Manning, Plowman et al. 2002).

1. Structural features of the AMPKs

AMPKs share a similar architecture with the Serine/Threonine kinase domain close to the N-terminus followed by a small regulatory domain, a long spacer region and, in most cases, a kinase-associated domain 1 (KA1) in C-terminus (figure 27) (Bright, Thornton et al. 2009; Marx, Nugoor et al. 2010; Moravcevic, Mendrola et al. 2010).

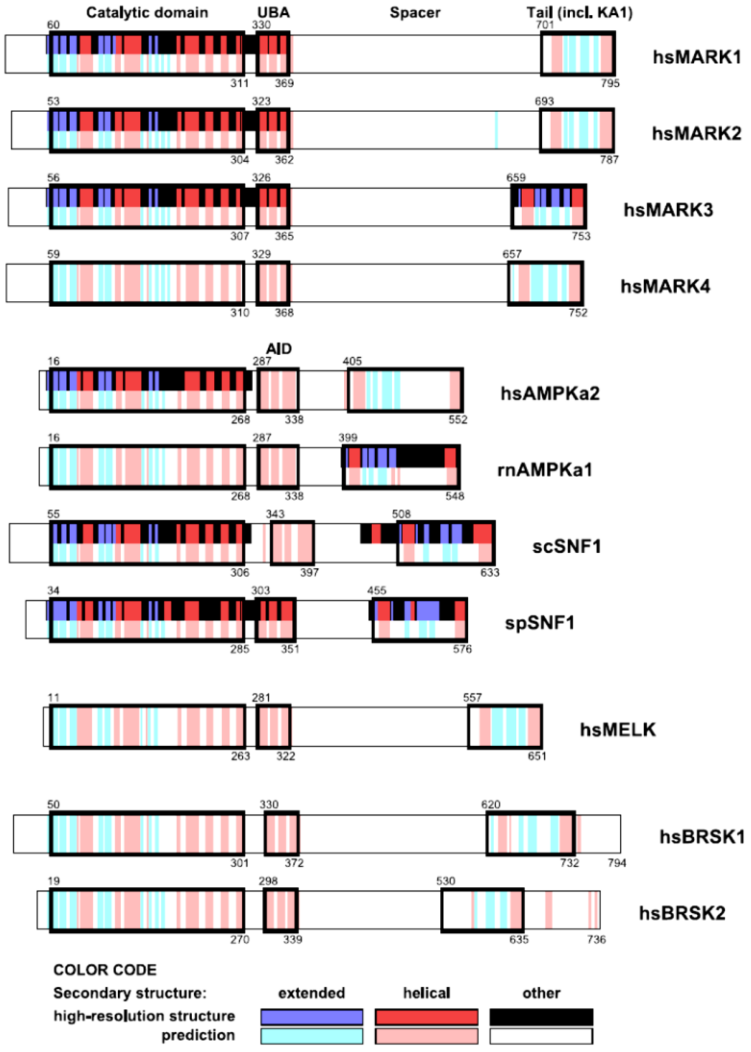


Figure 27: Secondary structure assignments and domain organization of MARK, AMPK, and AMPK-related kinases. (From (Marx, Nugoor et al. 2010)

Bars are colored according to secondary structure predictions using faint colors: cyan, extended (E); pink, helical (H); white, coil (C). For regions with known high resolution structure, secondary structure assignments based on these structures were determined with Procheck and added in the upper half of the bars, using intense colors: blue, extended strand (E); red, α-helices (H); black, other. Catalytic domains, autoregulatory domains (UBA or AID), and C-terminal tail domains (including the KA1 domain motif) are outlined by thick lines. Approximate domain limits are based on high-resolution structural information, either on structures of the domains themselves, if available, or on homologue structures. Boundaries of the catalytic domains are those of the UniProtKB sequence annotations, which are in good agreement with the structural data.

a) The kinase head

The overall fold of the catalytic domain of AMPK kinases is the same as that of many other protein kinases. It consists of a minor, N-terminal lobe (N lobe) and a larger C-terminal lobe (C lobe) with a cleft between them which has the ATP binding site and active site. The N lobe consists of 5-stranded β sheet and a single α helix (helix C), which is highly conserved and plays an important role in the regulation of many kinases. The C lobe is predominantly α-helical and comprises the T-loop, which is essential for the coordination of nucleotide and substrate in the catalytically active state.

The 2 lobes are linked by a flexible hinge region that allows opening and closing of the cleft. The hinge is a short peptide containing two glycines to provide flexibility. These two glycines are conserved in Cdr2. Almost all of the crystal structures of MARKs and AMPKs represent the inactive state where the N-lobe is tilted backwards, opening the cleft that is then blocked by the T-loop. Therefore the MARK and AMPK kinases have an open inactive conformation and a closed active conformation. The activity of the kinase domain of most MARKs and AMPKs is modulated at the level of two key elements, the T-loop and the UBA/AID domain as detailed below (Jaleel, Villa et al. 2006; Chen, Jiao et al. 2009; Marx, Nugoor et al. 2010).

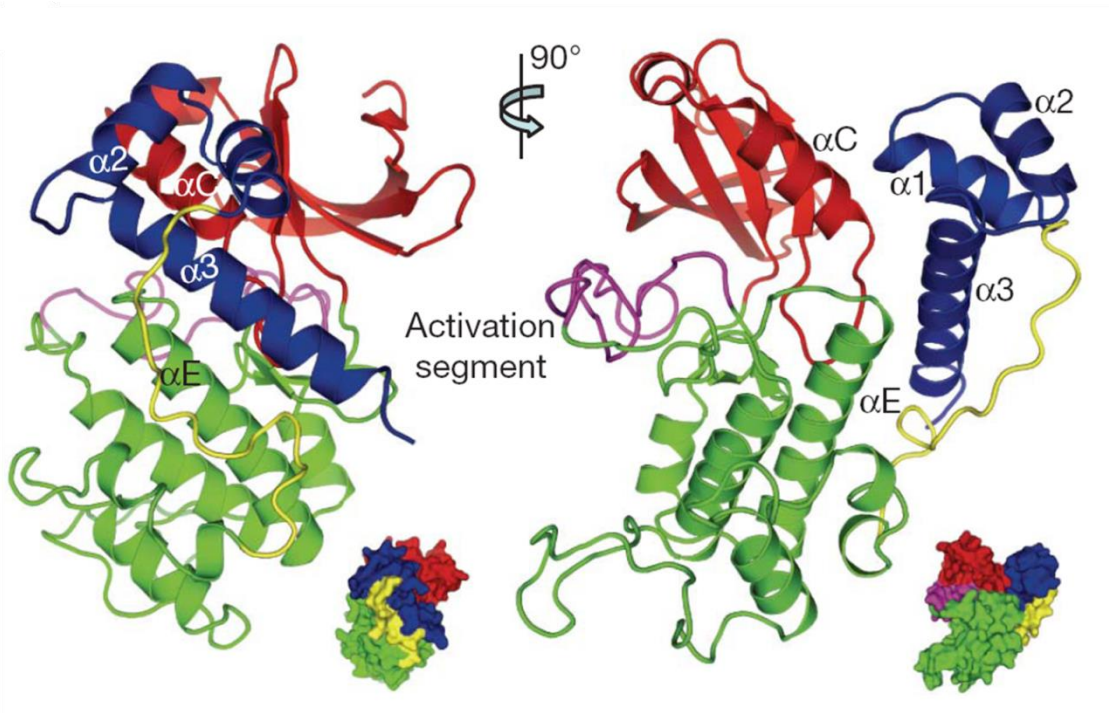


Figure 28: Schematic and surface representations of S. pombe KD-AID of pombe AMPK (From (Chen, Jiao et al. 2009))
(PDB: 3H4J) (in two views related by a 90u rotation around a vertical axis. The N- and C-lobes of the kinase domain are coloured in red and green, respectively, the activation segment is in magenta, the linker is in yellow, and the AID is in blue.

(1) The T-loop

The activation of AMPKs depends on the phosphorylation of a conserved threonine of the T loop by an upstream kinase. In humans, this phosphorylation depends LKB1 with the exception of MELK, which autophosphorylates. Phosphorylation of the T-loop by LKB1 increases the kinase activity at least 50 folds (figure 29) (Lizcano, Goransson et al. 2004). LKB1 functions in complex with STRAD pseudo kinase and MO25 (Hawley, Boudeau et al. 2003).

In *S. cerevisiae* the T-loop of the yeast ortholog of AMPK (SNF1) also needs to be phosphorylated. The phosphorylation is performed by a group of three related protein kinases homologous to LKB1 (Hong, Leiper et al. 2003; Nath, McCartney et al. 2003; Sutherland, Hawley et al. 2003).

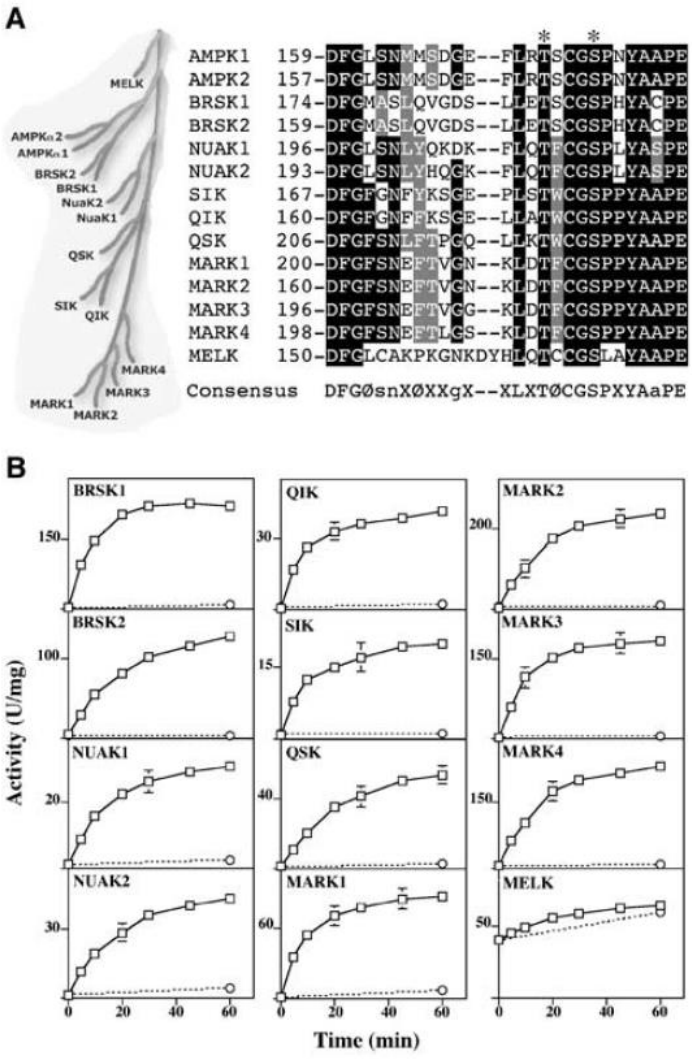


Figure 29: Activation of AMPK-related kinases by LKB1. (From (Lizcano, Goransson et al. 2004))

(A) Dendrogram and T-loop sequences of AMPK subfamily of protein kinases. The identical residues are shaded black and the conserved residues in grey. The T-loop Thr and Ser are indicated with an asterisk. (B) The indicated AMPK-related kinases were incubated with wild-type LKB1:STRAD:MO25 (open squares) or catalytically inactive LKB1[D194A]:STRAD:MO25 (open circles) complexes in the presence of Mg2þ and ATP. At the indicated times, the activity of the AMPK-related kinases was assayed with the AMARA substrate peptide, and the results are expressed as specific activity. Results shown are means7s.d. of assays carried out in triplicate and representative of two independent experiments. The error bars are only shown when larger than the size of the open squares. The suggested consensus sequence for optimal LKB1 phosphorylation is indicated. Ø represents a large hydrophobic residue; X, any amino acid; s, n, g and a preferences for Ser, Asn, Gly and Ala, respectively.

In *S.pombe,* the kinase that phosphorylates the T-loop of Cdr2 is the CAMK-related kinase Ssp1 (Deng, Baldissard et al. 2014). This kinase also activates the AMPK Ucp9/Ssp2 (Valbuena and Moreno 2012). Surprisingly, the T-loop threonine subject to phosphorylation is not conserved in Cdr1, suggesting that Cdr1 may be constitutively active.

There is a second phosphorylation site in the T-loop of AMPK kinases a serine located four residues downstream of the phosphor-threonine. Phosphorylation of this second residue has been shown to be less critical but can enhance the kinase activity (Lizcano, Goransson et al. 2004).

(2) The UBA/AID domain

AMPKs and MARKs share a small folded domain attached to the kinases domain either called UBA (UBiquitin Associated) due to sequence and structural similarity to ubiquitin associated domains, or AID (Auto Inhibitory Domain) linked to their function. Strikingly, these regulatory domains are either inhibitory as in the case of the catalytic subunit of AMPK, AMPKα1 (Chen, Jiao et al. 2009) or activator in the case of MELK (Beullens, Vancauwenbergh et al. 2005).

UBA and AID domains consist of a stretch of 40 amino-acids with low sequence homology. Nevertheless, crystal structures of Ucp9/Ssp2 AID domain associated with the kinase domain (KD+AID) on the one hand and of several Mark kinases UBA domains associated with their kinase domain (KD+UBA) in the other hand have been solved and revealed that both types of domains fold into a small globular structure of 3 α-helices that binds close to the back hinge of the kinase head between the N-lobe and C-lobe in the case of Ucp9 AID and on the N-lobe in the case of MARKs UBA. In both cases, direct interactions with Helix C of the N-lobe were found. Since helix C is a key element regulating the kinase activity, these interactions may be at the basis of the reported activatory or inhibitory functions of UBA/AID domains.

Based on comparison of crystal structures of UBA and AID domain, Marx et al. (2010) propose that the controversy between activatory and inhibitory functions of UBA and AID domains may be resolved if these domains serve as stabilizers of the open, inactive kinase domain conformation as well as the closed, active kinase domain conformation (Marx, Nugoor et al. 2010).

Sequence alignments and secondary structure predictions revealed that Cdr2 and Cdr1 have UBA/AID like domains. The function of Cdr2 UBA is studied in results section (Article 2).

Cdr1 UBA is functionally important as it has been shown to bind the Cdr1 inhibitors Nif1 (Wu and Russell 1997) and Skb1 (Deng and Moseley 2013). In both cases the binding site has been mapped by Y2H and narrowed down to the region corresponding to Cdr1 UBA. How Nif1 and Skb1 binding to Cdr1 UBA inhibits Cdr1 functions remains unknown.

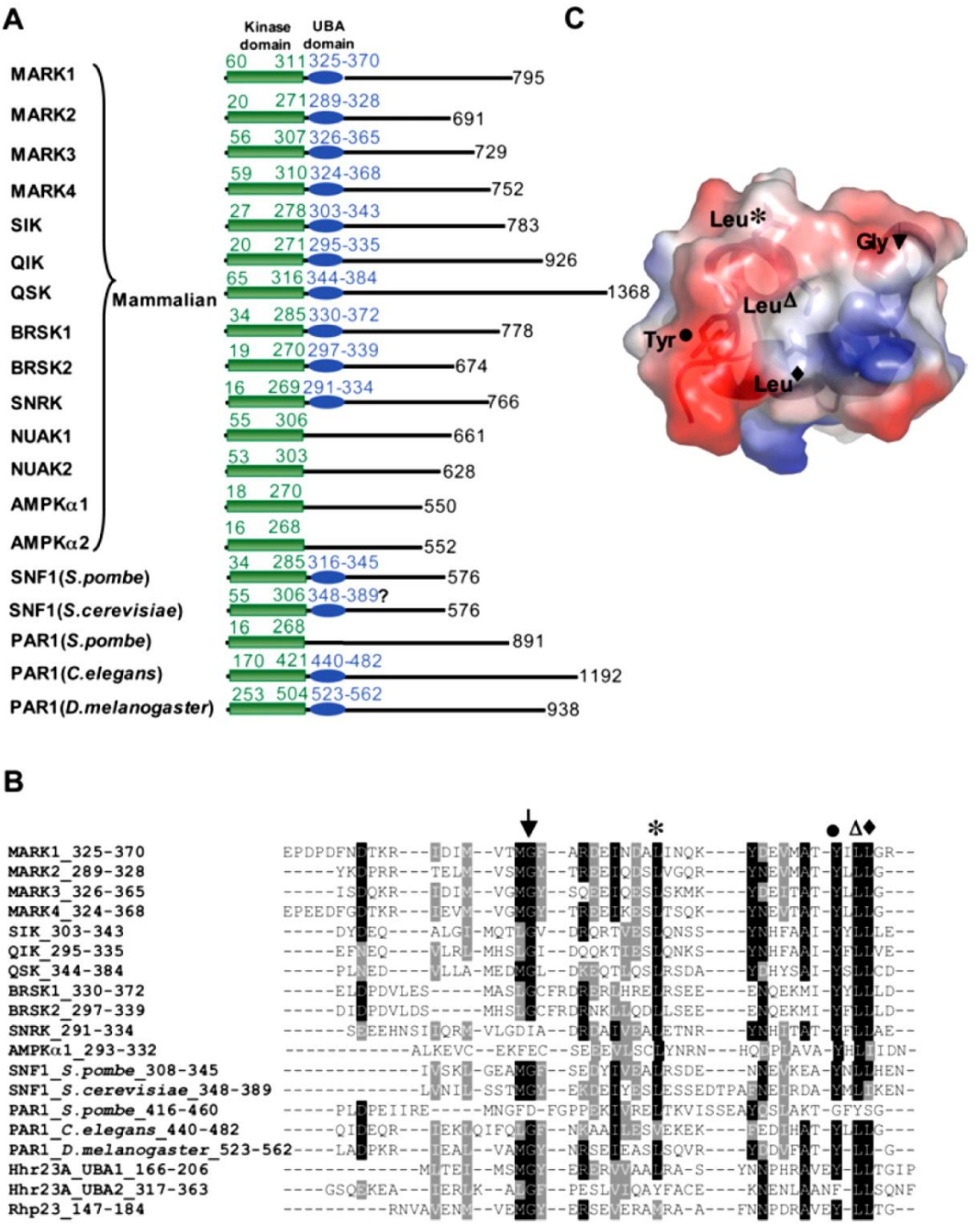


Figure 30: Presence of the UBA domain in AMPK-related kinases. (From (Jaleel, Villa et al. 2006)

(A) Schematic representation of the 14 mammalian AMPK subfamily kinases that are activated by LKB1 as well as the homologues of these enzymes in the indicated species. The presence or absence of the UBA domain was determined employing the ScanProsite (http://us.expasy.org/tools/scanprosite/) and SMART (Simple Modular Architecture Research Tool) programs (http://smart.embl-heidelberg.de/). The residues encompassing the catalytic and UBA domains are in green and blue respectively. The number of residues present in each kinase is also indicated in black. Neither SMART or ScanProsite program recognizes the putative UBA domain indicated in S. cerevisiae SNF1, which is, therefore, indicated with '?'. (B) The alignment of the UBA domain sequences of AMPK subfamily protein kinases with UBA domains of Hhr23A and Rhp23.

b) The KA-1 domain

AMPKs share a C-terminal domain called KA-1 (Kinase associated domain) the function of which remained elusive for a long time. In 2010 Moravcevic and collaborators showed that KA-1 domains represent a type of lipid binding domain with highest affinity for the acidic phospholipid phosphatidyl serine (Moravcevic, Mendrola et al. 2010). They provided crystal structures of the MARK1 KA-1 and *S. Cerevisiae* Kcc4 KA-1 domains. These structures highlighted that interactions with lipids relies on a series of basic residues located on the surface of the domain that establish electrostatic interactions with acidic phospholips. In addition, a hydrophobic loop was proposed in insert in the lipid bilayer in the case of Kcc4. However, this loop is not conserved in MARKs (Moravcevic, Mendrola et al. 2010) nor in fission yeast Cdr2 (Rincon, Bhatia et al. 2014).

As mentioned earlier, Cdr2 KA1 domain has a similar function in lipid binding but has an additional clustering property that depends on an hydrophobic loop lying on the side of the domain opposite to the membrane binding surface. Lipid binding is also reinforced by a basic domain upstream of Cdr2 KA-1 (Rincon, Bhatia et al. 2014).

Surprisingly enough Cdr1, which also belongs to the AMPK family of kinase, does not have a KA1 and completely depends on Cdr2 to bind the cell cortex.

(1) The tail

The tail domain correspond to the very last aminoacids after the KA1 domain found in most AMPK kinases. It has been proposed that the tail domain could also have a role in some kinases as a regulatory domain having an impact on kinase activity by directly binding the kinase domain in a functionally similar way as UBA/AID domains would do. This hypothesis is based on the observation that when the tail domain of MELK is removed a 2-4 fold increase on kinase activity is observed. These observations are consistent with the assumption that an intact tail domain is required for effective autoinhibition of MELK. An autoinhibitory function of the tail

(requiring the presence of the KA1 domain) has also been reported for Kin1/2, the MARK/Par-1 orthologs of *S. cerevisiae* (Beullens, Vancauwenbergh et al. 2005; Elbert, Rossi et al. 2005).

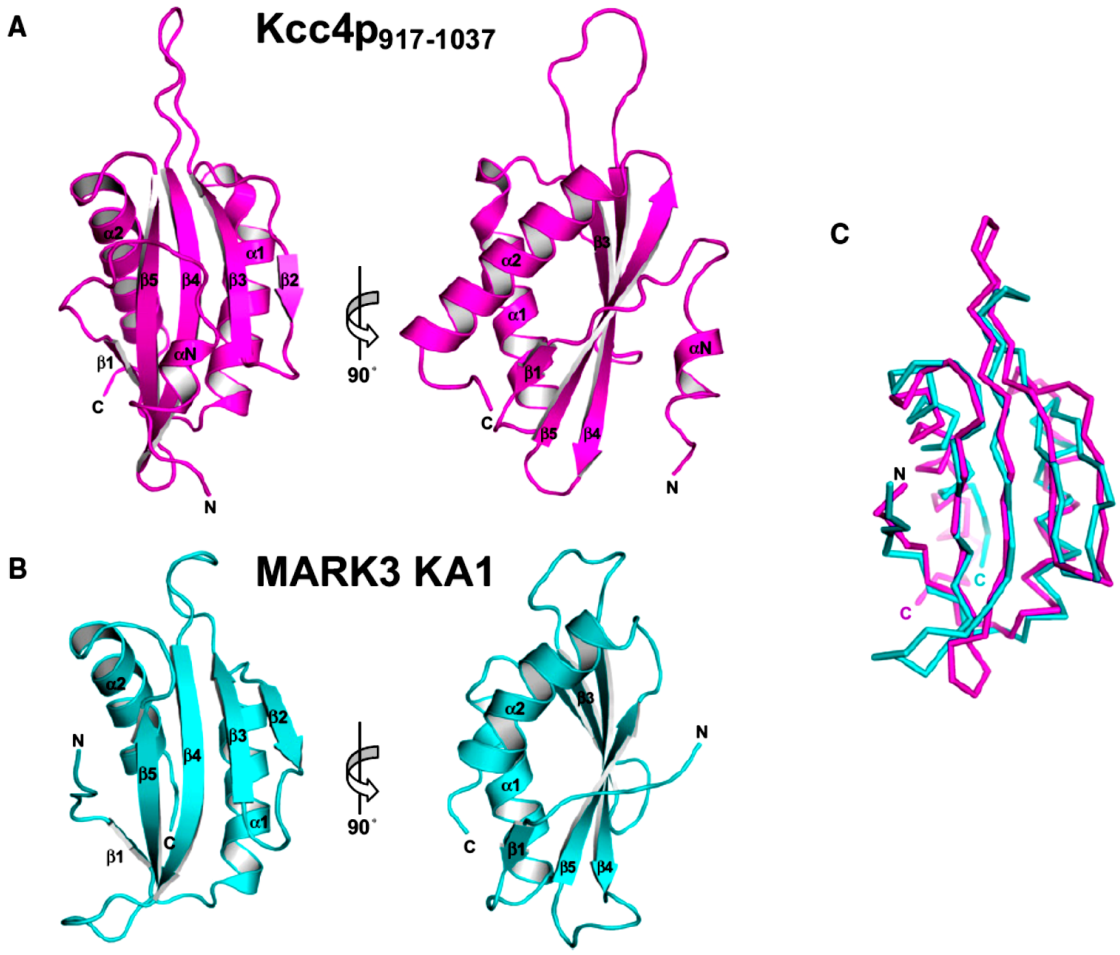


Figure 31. The Kcc4p C Terminus Adopts a KA1 Domain Fold. (From (Moravcevic, Mendrola et al. 2010))

(A) Cartoon representation of Kcc4p917–1037 structure. Helices aN, a1, and a2 are marked, as are strands b1–b5. Two orthogonal views are shown. (B) NMR structure of the KA1 domain from mouse MARK3 (PDB ID 1UL7), in the same orientations used in (A) for Kcc4p917–1037. (C) Cα overlay of MARK3-KA1 (cyan) with Kcc4p917–1037 (magenta). The N-terminal part of Kcc4p917–1037, including helix aN, was removed for clarity

III.

Results

III. Results

A. Role of Blt1 in stabilizing the ring precursor nodes during their maturation and compaction

1. Bakground

During interphase the anillin-like protein Mid1, which is the major division plane factor of fission yeast, localizes to the medial cortex to predefine the division plane (Sohrmann, Fankhauser et al. 1996; Paoletti and Chang 2000; Celton-Morizur, Bordes et al. 2004). This localization is controlled by the CGN (cell genometry network) composed of medial cortical nodes of Cdr2 that serves as a membrane receptor for Mid1 and of Pom1 polarity kinase that forms gradients emanating from the cell tips which restrict the Cdr2 nodes assembly to the medial cortex. This mechanism ensures cell partitioning in two equal halves during cytokinesis (Bahler and Pringle 1998; Celton-Morizur, Racine et al. 2006; Padte, Martin et al. 2006; Moseley, Mayeux et al. 2009; Almonacid, Celton-Morizur et al. 2011).

A parallel mechanism links the division plane to nuclear position at mitotic entry. This mechanism depends on the fast export of Mid1 nuclear pool at mitotic entry, induced by the polo-like kinase Plo1 (Bahler and Pringle 1998; Almonacid, Celton-Morizur et al. 2011). This phosphorylation also activates Mid1, which initiates the assembly of the contractile ring from medial cortical nodes by recruiting the IQGAP Rng2 which in turn promotes Myosin II recruitment (Almonacid, Celton-Morizur et al. 2011; Laporte, Coffman et al. 2011; Padmanabhan, Bakka et al. 2011).

Besides anchoring to the Cdr2 nodes, Mid1 can directly anchor to the membrane thanks to a lipid-binding amphipathic helix that may insert in the lipid bilayer (Celton-Morizur, Bordes et al. 2004). In cells where the membrane anchoring domain of Mid1 is mutated, Mid1 only relies on Cdr2 nodes for cortical anchoring. This pathway is sufficient for contractile ring assembly in the cell middle in the vast majority of cells (Celton-Morizur, Bordes et al. 2004; Almonacid,

Moseley et al. 2009). However, Cdr2 starts dissociating from the nodes at the G2/M transition when Mid1 needs proper membrane anchoring to recruit essential ring components to the cortex for contractile ring assembly. This suggests that Mid1 may associate with another membrane-bound factor in early mitosis. Accordingly, in the study identifying Cdr2 as a receptor for Mid1, a systematic internal deletion approach identified a region comprising Mid1 aa 300 to 350 as critical for Mid1 cortex anchoring in absence of an amphipathic helix. One proposed explanation was that this region may represent a binding site for the second membrane anchor protein for Mid1.

In parallel to this study, a series of new components of medial cortical nodes were identified, including Blt1. Blt1 was initially identified as a binding partner of the F-Bar contractile ring protein Cdc15. Blt1 was shown to localize the cortex in interphase a more complex pattern than Cdr2, in a series of nodes at one cell tip in very early G2 that were gradually replaced by medial nodes coinciding with Cdr2 nodes later during G2. Accordingly, Blt1 was found to lose its medial localization in the absence Cdr2 but remained associated with the cortex at one cell tip, similar to Blt1 distribution in short G2 cells. Two additional components of the nodes, Klp8 and Gef2 were shown to associate with Cdr2 nodes through Blt1. During mitosis, Blt1 compacted with other node components in the contractile ring and remained associated with it throughout cytokinesis. This suggested that Blt1 may have a cytokinetic role although no division plane defects were observed in its absence. In contrast, Blt1 deletion led to slightly longer cell size at division than in the *wt* situation suggesting it may modulate the Cdr2-dependent Wee1 regulatory pathway organized by Cdr2 in medial cortical nodes (Moseley, Mayeux et al. 2009).

In the first part of my thesis, I studied the function of Blt1 in collaboration with the group of James Moseley. In this study, we identified Blt1 as the membrane-binding factor interacting

with Mid1 (300-350) domain. We showed that this interaction was indirectly mediated by Gef2 that may be the true binding partner of Mid1 300-350 domain (Ye, Lee et al. 2012). We found that Blt1 possesses a basic C-terminal membrane anchoring motif that stabilizes the ring precursor nodes on the medial cortex in early mitosis in parallel to Mid1 lipid binding.

Article 1: Blt1 and Mid1 Provide Overlapping Membrane Anchors To Position the Division Plane in Fission Yeast

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Blt1 and Mid1 Provide Overlapping Membrane Anchors To Position the Division Plane in Fission Yeast

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Spatial control of cytokinesis is essential for proper cell division. The molecular mechanisms that anchor the dynamic assembly and constriction of the cytokinetic ring at the plasma membrane remain unclear. In the fission yeast *Schizosaccharomyces pombe*, the cytokinetic ring is assembled in the cell middle from cortical node precursors that are positioned by the anillin-like protein Mid1. During mitotic entry, cortical nodes mature and then compact into a contractile ring positioned in the cell middle. The molecular link between Mid1 and medial cortical nodes remains poorly defined. Here we show that Blt1, a previously enigmatic cortical node protein, promotes the robust association of Mid1 with cortical nodes. Blt1 interacts with Mid1 through the RhoGEF Gef2 to stabilize nodes at the cell cortex during the early stages of contractile ring assembly. The Blt1 N terminus is required for localization and function, while the Blt1 C terminus promotes cortical localization by interacting with phospholipids. In cells lacking membrane binding by both Mid1 and Blt1, nodes detach from the cell cortex and generate aberrant cytokinetic rings. We conclude that Blt1 acts as a scaffolding protein for precursors of the cytokinetic ring and that Blt1 and Mid1 provide overlapping membrane anchors for proper division plane positioning.

Cell division requires the spatial and temporal coordination of many cellular activities. During cytokinesis, the final act of the cell cycle, a contractile actomyosin ring constricts to separate the two daughter cells. The contractile ring must be properly assembled and positioned to ensure equal segregation of cellular materials to each daughter cell. The contracting cytokinetic ring maintains association with the cell cortex, which undergoes dramatic remodeling and membrane bending during this process. The mechanisms that position and anchor components of the cytokinetic ring in the plasma membrane have been the subject of intense study, as defects in this process can lead to a range of cellular defects and disease states (1–4).

Many insights into eukaryotic cytokinesis have come from work on the fission yeast *Schizosaccharomyces pombe* (1, 5). These rod-shaped cells grow in a linear manner at the cell ends and then position the contractile ring precisely in the cell middle at division. This positioning occurs through the combination of inhibitory signals emanating from the cell ends and positive cues from the nucleus in the cell middle. These positional cues act largely through the protein Mid1, which is similar to anillin in metazoans (6, 7). During interphase, Mid1 localizes to the nucleus and to a band of cortical nodes that are positioned in the cell middle. These interphase nodes are organized by the protein kinase Cdr2 and are spatially restricted to the cell middle by inhibitory cues from the cell tips (8–12). During interphase, these cortical nodes also contain a cell cycle regulatory network that couples mitotic entry with cell size (11, 13). Proteomic studies have revealed additional node components, such as the protein Blt1, the RhoGEF Gef2, and the kinesin Klp8 (11), which remain largely uncharacterized. Blt1, Gef2, and Klp8 all localize to cortical nodes and to the contractile cytokinetic ring, but their absence does not lead to obvious cytokinetic defects. This suggests the possibility that they function redundantly with other cytokinetic proteins.

As the cell enters mitosis, many cytokinesis proteins, including type II myosin and actin-binding proteins, are recruited to medial cortical nodes, which subsequently condense to form the contractile ring (5). Mid1 is required for the localization of cytokinesis proteins to these cortical nodes (14–21), and *mid1* mutants display severely misplaced contractile rings and septa (6, 7). These findings indicate that recruitment of Mid1 to cortical nodes is a key step in the assembly and positioning of cytokinesis. However, the molecular mechanisms that anchor Mid1 at cortical nodes in the plasma membrane remain unclear. The carboxyl terminus of Mid1 contains a membrane-binding amphipathic helix, but deletion of this helix does not greatly impair Mid1 localization or function. Rather, the amino-terminal half of Mid1 (Mid1-Nter; residues 1 to 506) is necessary and largely sufficient for Mid1 function and localization to cortical nodes (22).

In this study, we found that the node protein Blt1 is required for the localization and function of Mid1-Nter. Further, the interaction of Blt1 with Mid1 is mediated by the RhoGEF Gef2. Using a structure-function approach, we identified an N-terminal domain of Blt1 that is necessary for localization to medial cortical nodes and function. A separate C-terminal membrane-binding domain functions in parallel to the Mid1 membrane-binding helix to anchor Mid1 at the medial cortex and promote cell division in the cell middle. Our findings indicate that the assembly of the cytokinetic ring by cortical precursors requires multiple interactions of cortical node components with the plasma membrane.

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MATERIALS AND METHODS

Strains and plasmids. All *S. pombe* strains used were isogenic to 972 and are listed in Table S1 of the supplemental material. Standard *S. pombe* molecular genetics techniques and media were used (23). Strains were selected from genetic crosses by random spore analysis or tetrad dissection.

The Mid1 plasmids used in this study were derived from integrative vector pJK148 (24). pAP93, pAP146, pAP159, pSM26, pMA32, and pMA34 were described previously (8, 22, 25). Of note, pAP93 (pmid1-mid1) contains a point mutation at amino acid (aa) 6 of Mid1, which does not alter Mid1 function (A. Paoletti, unpublished data). pMA15 (pmid1-GFP-mid1-300-350) was obtained by fusing a NotI-BamHI PCR product containing Mid1 aa 300 to 350 to pSM26 (containing pmid1 and the green fluorescent protein [GFP] gene) cut with similar enzymes. pMG60 (pmid1-GST-mid1-300-450) was obtained by subcloning a XhoI-NotI fragment containing the glutathione *S*-transferase (GST) gene from pDS473 (a generous gift from S. Forsburg) and a NotI-SacI fragment from pMA16 (8), containing Mid1 aa 300 to 450 followed by the nmt1 stop, into pAP140 (22), which had been cut by SalI and SacI. Plasmids were linearized by NruI in the *leu1* gene and then integrated into the genome of *mid1*Δ *leu1-32*, *mid1*Δ *blt1*Δ *leu1-32*, *mid1*Δ *blt1-mEGFP leu1-32*, and *mid1*Δ *blt1-mCherry leu1-32* strains, due to the tight genetic linkage between *leu1* and *blt1* loci. Transformations were performed using the lithium acetate-dimethyl sulfoxide method (26).

To introduce the Mid1-Nter construct at the *mid1* locus, 500-bp fragments corresponding to the end of the Mid1 N terminus (aa 1 to 506) and *mid1* terminator (*tmid1*) were amplified by PCR using oligonucleotides terminated by sequences specific for the pFa6a-hphMX6 plasmid (27). These fragments were used to produce a PCR product encoding *mid1-Nter-tADH-NatMX-tmid1* by using Primestar enzyme (TaKaRa). This PCR product was integrated into strains AP3906 and AP3907 to produce strains AP3924 and AP3925.

An integration plasmid for Cdr2 constructs was produced by insertion of 1 kb of the *cdr2* promoter (*pcdr2*) and terminator (*tcdr2*) into pFA6a-GFPkanMX6 (26) between SalI and BamHI sites and SacI and SpeI sites, respectively. The *cdr2*$^+$ open reading frame (ORF) was then inserted upstream of GFP(S65T) between BamHI and PacI to create pSR34. Finally, GFP was replaced between PacI and AscI sites by GFP-CAAX, amplified by PCR by using a reverse oligonucleotide encoding the last 19 amino acids of Mod5, including a prenylation motif (KPPKKKGSKLEKFCCILM [28]) in frame with GFP. NotI fragments of pSR34 and pSR58 containing *pcdr2-cdr2-GFP-tADH-kanMX-tcdr2* or *pcdr2-cdr2-GFP-CAAX-tADH-kanMX-tcdr2*, respectively, were transformed in a *cdr2*Δ::*natMX6 leu1-32* *h*$^-$ strain (AP2804) to produce strain AP3177 and AP3909. Geneticin-resistant and CloNat-sensitive clones were checked by PCR for proper genome integration of Cdr2 constructs.

To map Blt1 localization domains, Blt1 fragments were PCR amplified and subcloned into the NdeI-BamHI sites of pREP41-GFPN (29). These plasmids were transformed into strain JM429; overexpression was induced by growth in medium lacking thiamine for at least 24 h at 25°C.

To integrate *blt1*Δ*1*-mCherry, a fragment containing *pblt1-blt1-mCherry-tadh1* was PCR amplified from strain JM1598 and subcloned into integrative vector pJK210. The StuI sites in mCherry were removed by silent mutations using site-directed mutagenesis. The coding sequence for Blt1 amino acids 2 to 78 was deleted by site-directed mutagenesis to generate *blt1*Δ*1*-mCherry. Both the full-length pJK210-*pblt1-blt1-mCherry-tadh1* (pJM584) and the truncated pJK210-*pblt1-blt1*Δ*1-mCherry-tadh1* (pJM585) plasmids were linearized by digestion with StuI, and then integrated into the *ura4-294* allele. The resulting integrants were combined with *blt1*Δ::natR, meaning that Blt1-mCherry or *blt1*Δ*1*-mCherry was expressed as the sole genomic copy and under the control of the endogenous promoter. To generate blt1Δ5 mutants, we integrated mCherry-natR or GFP-kanMX6 at the endogenous *blt1*$^+$ locus after the codon for amino acid 575 by using the pFA6a system (26).

Coimmunoprecipitation and lipid binding experiments. For coimmunoprecipitations, 200 ml of cells were grown to an optical density at 595 nm of 1 at 30°C in YE5S medium concentrated 2 times compared to regular YE5S medium (YE5S2×). The cells were first washed with 1 ml of Stop buffer (NaCl at 150 mM, NaF at 50 mM, NaEDTA at 10 mM, NaN_3 at 1 mM), then resuspended in 600 μl 1D buffer (HEPES at 50 mM [pH 7.5], NaCl at 100 mM, EDTA at 1 mM, NP-40 at 1%, β-glycerophosphate at 20 mM, NaF at 50 mM, Na_3VO_4 at 0.1 mM, phenylmethylsulfonyl fluoride at 1 mM complemented with complete EDTA-free antiprotease tablets [Roche]) together with 600 μl of glass beads and broken using a FastPrep FP120A instrument (Qbiogene; two cycles of 40 s at maximum speed). Lysates were then spun at 10,000 × *g* for 10 min at 4°C, and supernatants were recovered. Soluble extracts were incubated with anti-mouse IgG magnetic beads (M-280 Dynal; Invitrogen) coupled to 6 μg of anti-GFP monoclonal antibody (MAb; Roche), antihemagglutinin (anti-HA) MAb 12CA5 (Roche), or anti-myc MAb 9E10 (Roche) for 2 h at 4°C; then, the beads were washed five times with 1D buffer and the beads were resuspended in SDS-PAGE sample buffer. Immunoprecipitation (IP) samples and soluble extracts were submitted to SDS-PAGE and transferred to nitrocellulose membranes. Western blot assays were performed with anti-GFP MAb (1/500; Roche), and anti-Mid1 affinity-purified Ab (1/200 [22]). Secondary antibodies were coupled to peroxidase (Jackson ImmunoResearch) or to alkaline phosphatase (Promega). Signal quantification was performed in Metamorph. The signals of coimmunoprecipitated proteins were normalized relative to the protein concentration in the input and the amount of primarily precipitated protein.

For lipid-binding assays, GFP-Blt1- and GFP-blt1Δ5-containing extracts were prepared by bead beating in lysis buffer (1× Tris-buffered saline, 0.5% Triton X-100, 1 mM EDTA, protease inhibitor cocktail [Roche]) and clarified by centrifugation at 16,000 × *g* for 10 min at 4°C. Clarified extracts were diluted in blocking buffer and incubated with membrane lipid strips (Echelon Bioscience) according to the manufacturer's protocol. Lipid strips were probed with anti-GFP antibodies (11).

Microscopy. Cells were grown exponentially at 25°C in YE5S or EMM4S, except for the cells shown in Fig. S1 in the supplemental material, which were grown overnight in patches on YE5S plates to limit autofluorescence. Cells were imaged in liquid medium under a coverslip using four microscopes, as follows.

For the images in Fig. 9 and Fig. S1, S3, and S11 in the supplemental material, microscopy was performed on a DMRXA2 upright microscope (Leica Microsystems) equipped with a 100×/1.4-numerical-aperture (NA) Plan Apochromat objective and a Coolsnap HQ charge-coupled-device (CCD) camera (Roper). In the image in Fig. S3, 5 interphase cells of similar lengths (12.9 to 13.5 μm) were randomly selected from differential interference contrast (DIC) images. GFP fluorescence along the cortex from tip to tip was analyzed by using the line scan tool of Metamorph software (4 pixels in width). Background values were subtracted before plotting.

For the images in Fig. 1A and 8A and Fig. S2, S9, and S10 in the supplemental material, we used a Nikon Eclipse TE2000-U microscope equipped with a 100×/1.45-NA oil immersion objective, a PIFOC objective stepper, a Yokogawa CSU22 confocal unit, and a Roper HQ2 CCD camera. For time-lapse movies in Fig. 8A and Fig. S10 in the supplemental material, stacks of 7 planes spaced by 1 μm were acquired every 2 min (binning 2, gain 3; 500 ms at 15% laser power for both GFP and mCherry [Fig. 8A], or 20% of GFP laser power [Fig. S11]). In Fig. 1A and also Fig. S2 and S9 in the supplemental material, individual stacks were taken using a laser power of 60% for GFP and 80% for mCherry, and 13 planes were taken with a step size of 0.5 μm and 300-ms exposure.

For Fig. 1B, 3, 4, 6, and 7 and Fig. S5 to S7 in the supplemental material, images were obtained on a DeltaVision imaging system and processed by iterative deconvolution as described previously (30).

For Fig. 5, cells were imaged by spinning disk confocal microscopy as described previously (30). Images were analyzed in ImageJ or Metamorph.

RESULTS

Blt1 is required for division plane positioning when Mid1 binding to membranes is impaired. The N-terminal half of Mid1 localizes to the nucleus, cortical nodes, and the cytokinetic ring and division septum (22). Cells expressing Mid1-Nter lack the C-terminal half of Mid1, making them functionally dependent on interactions made by Mid1-Nter. The localization of Mid1-Nter to medial cortical nodes and its function are fully disrupted in *cdr2*Δ cells, which lack cortical nodes in the cell middle. Similar defects are observed when a Mid1-Nter site necessary for Mid1 interaction with Cdr2 is deleted (aa 400 to 450). Interestingly, deletion of another region of Mid1-Nter (aa 300 to 350) yields the same phenotype without impairing the Mid1-Cdr2 interaction (8), suggesting that other components of cortical nodes may be required for the cortical recruitment and function of Mid1-Nter.

Since Blt1 and Mid1 coimmunoprecipitate in cell extracts (11) and Blt1 colocalized with Mid1-Nter in cortical nodes during interphase and at the cytokinetic ring (see Fig. S1 in the supplemental material), we tested the possibility that Blt1 recruits Mid1-Nter to cortical nodes. We found that in the absence of Blt1, Mid1-Nter localized to the nucleus but was largely absent from cortical nodes (Fig. 1A and B). These Mid1-Nter *blt1*Δ cells displayed misplaced cytokinetic rings and septa similar to *mid1*Δ (Fig. 1A). Only a small proportion of Mid1-Nter and *blt1*Δ single mutant cells displayed misplaced septa, consistent with previous results (11, 22), but septa were misplaced in nearly all Mid1-Nter *blt1*Δ double mutant cells (Fig. 1C). This synthetic defect indicated that Blt1 functions to recruit Mid1 to the cell cortex for cytokinesis in the absence of the Mid1 C terminus.

The C terminus of Mid1 contains an amphipathic helix that associates with membranes at the cell cortex. Mutation of this helix (*mid1-helix**) leads to only minor cytokinesis defects similar to those resulting from deletion of the entire C terminus in the Mid1-Nter construct. We hypothesized that this amphipathic helix and Blt1 represent overlapping mechanisms that attach Mid1 to the cell cortex. Consistent with this model, combining *mid1-helix** and *blt1*Δ mutations led to severe defects in the position of division septa (Fig. 1C). We concluded that Mid1 associates with the cortex to position the cytokinetic ring through both its amphipathic helix and interaction with Blt1 and Cdr2 at medial cortical nodes.

Mid1(300-350) mediates interaction with Blt1 through Gef2. We next investigated the physical interaction between Mid1 and Blt1 at the cell cortex. Deletion of Mid1 300-350 dramatically reduced the Mid1 interaction with Blt1 (Fig. 2A), indicating that residues 300 to 350 of Mid1 are required for association with Blt1. Indeed, deletion of these residues in Mid1-Nter abolished localization to cortical nodes and the contractile ring (see Fig. S2 in the supplemental material), as previously reported (8, 31). Interestingly, we also found that Blt1-mEGFP was less concentrated in the cell middle in *mid1*Δ*300-350* cells (see Fig. S3 in the supplemental material), suggesting that the interaction of Mid1 and Blt1 may reciprocally promote their localization to medial cortical nodes.

Recruitment of Mid1-Nter to cortical nodes also depends on Gef2 (31), a putative Rho GTPase GEF protein. Similar to Mid1-Nter, the localization of Gef2 to interphase cortical nodes requires Blt1 (11, 31). Thus, we considered that Gef2 might bridge the interaction between Blt1 and Mid1-Nter. Indeed, coimmunoprecipitation of Blt1 and Mid1 was abolished in *gef2*Δ cells (Fig. 2B).

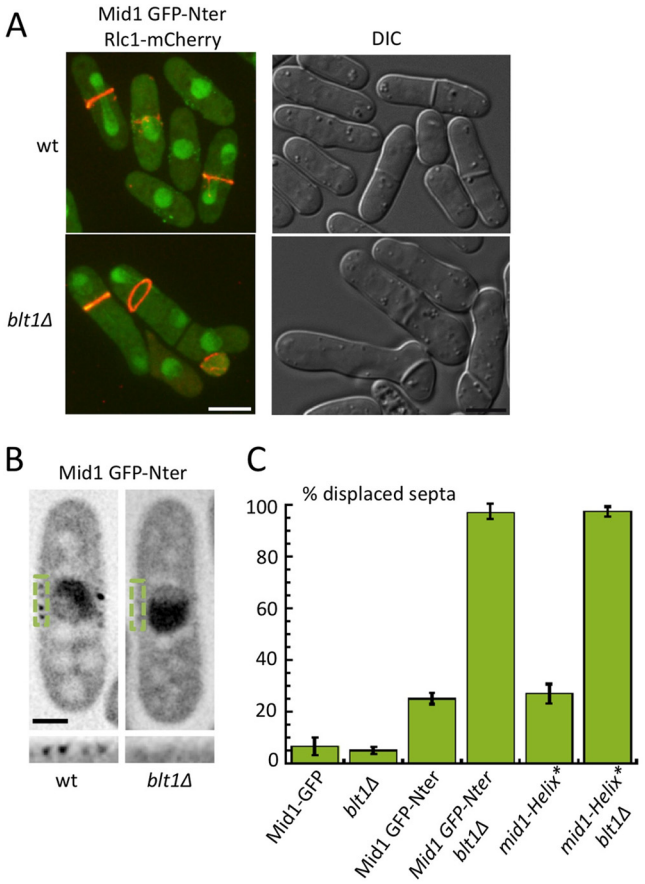

FIG 1 Blt1 promotes Mid1-Nter localization and function. (A, left) Localization of the Mid1 N terminus (Mid1 GFP-Nter) and Rlc1-mCherry in wild-type and *blt1*Δ cells deleted for endogenous *mid1*. Strains used were AP3900 and AP3903. Images are maximum projections of spinning disc confocal *z*-series. (Right) DIC images of the same mutants. Strains were AP998 and AP2172. Bar, 5 μm. (B) Mid1 GFP-Nter is largely absent from cortical nodes in *blt1*Δ cells. Images are deconvolved inverted single focal planes, and the bottom row shows magnified views of green, boxed regions. Bar, 2 μm. Strains used were AP998 (4 nodes/cell; $n = 77$ cells) and AP2172 (0.5 node/cell; $n = 64$ cells). (C) Percentage of displaced septa for the indicated genotypes (strains AP528, AP998, JM429, AP2172, AP583, and AP2335). Bars represent means ± standard deviations (error bars) from two separate experiments ($n > 300$ cells in each experiment).

In contrast, Gef2 coimmunoprecipitated with Mid1 in both wild-type and *blt1*Δ cells (Fig. 2C). This suggested that Blt1 associates with Mid1-Nter indirectly through Gef2, which may act as an adaptor protein in cortical nodes.

To further investigate these physical interactions, we identified by coimmunoprecipitation a minimal fragment of Mid1 that associated with Blt1 and Gef2 (see Fig. S4A in the supplemental material). Similar to the full-length protein, Mid1(300-450) associated with Blt1 in wild-type but not *gef2*Δ cell extracts, while the same fragment interacted with Gef2 independently of Blt1 (see Fig. S4B and C). Furthermore, we found that Mid1 residues 300 to 350 were sufficient for localization to the cytokinetic ring and septum in cells, and this localization was lost in *blt1*Δ cells (see Fig. S2C and D in the supplemental material). These combined data indicate that residues 300 to 450 of Mid1 interact with Blt1 indirectly through Gef2 to promote Mid1-Nter cortical localization,

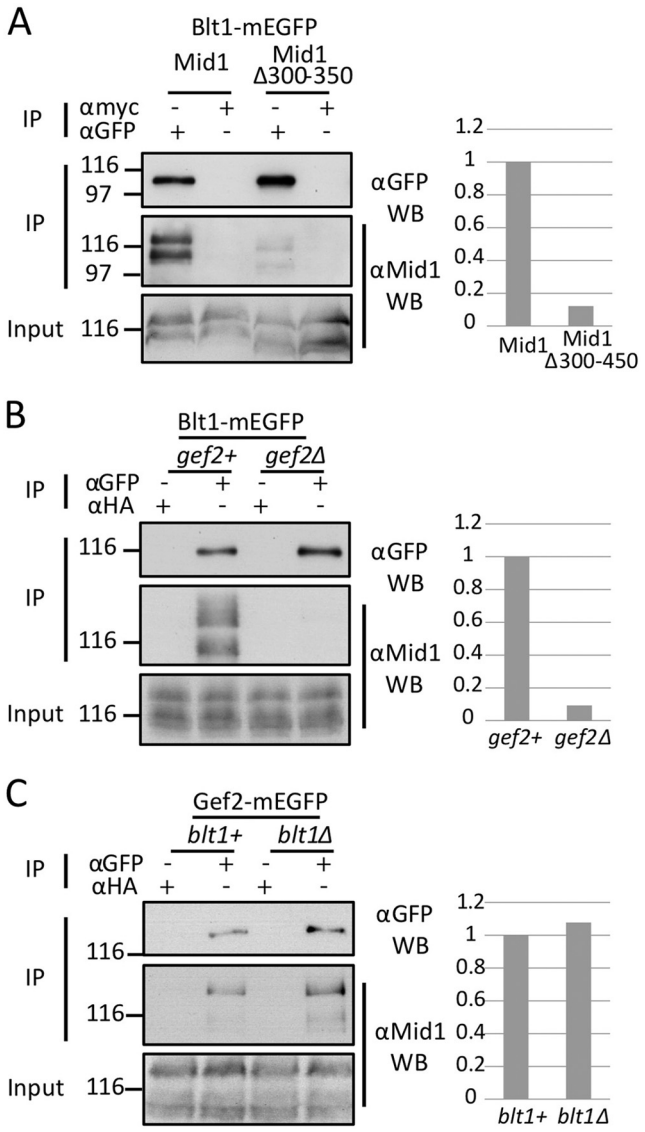

FIG 2 Mid1 interacts with Blt1 through Gef2. (A) Coimmunoprecipitation assay results between Blt1-mEGFP and the indicated Mid1 construct. Immunoprecipitation was performed with an anti-GFP MAb, or with an anti-HA or anti-myc MAb as negative controls. Input and IP samples were probed with anti-Mid1 antibodies. Normalized signal quantification from two independent experiments is shown on the right. Strains used were AP3490 and AP3491. (B and C) Coimmunoprecipitation assay results between Blt1 (B) or Gef2 (C) and Mid1 in *gef2*$^+$ or *gef2*Δ or *blt1*$^+$ *or blt1*Δ backgrounds, respectively. Immunoprecipitation was performed as for panel A. Normalized signal quantification from two independent experiments is shown on the right. Strains used were JM151, AP3872, JM365, and AP3873.

contractile ring localization, and function. This raises the possibility that Blt1 acts as a cortical anchor for Mid1-Nter, with Gef2 functioning to bridge Blt1-Mid1 interactions either directly or indirectly.

Blt1 associates with the cell cortex. Based on our evidence that Blt1 anchors Mid1-Nter at cortical nodes, we hypothesized that Blt1 is a lipid-binding scaffold protein for cortical nodes and the contractile ring at the plasma membrane. Indeed, Blt1 localizes to cortical structures (nodes and contractile ring) throughout the cell cycle, and it also remains at the cell cortex upon disruption of nodes by *cdr2*Δ (11). In support of Blt1 as a membrane scaffold, we observed Blt1-mEGFP in a contractile ring that was external of the contractile myosin ring, marked by Rlc1-mRFP, during cytokinesis (see Fig. S5A and B in the supplemental material). This organization was present in all cells examined ($n = 20$) and was maintained when we imaged Blt1-mCherry Rlc1-mEGFP cells, indicating that it was not due to the respective fluorophores (see Fig. S5A). This places Blt1 in a position to link the membrane with components of the contractile ring.

To investigate the Blt1 association with the cell cortex further, we increased the expression of GFP-Blt1. When overexpressed by the strong P3nmt1 promoter, GFP-Blt1 coated the cell periphery (see Fig. S5C and D in the supplemental material). This indicated that Blt1 association with the cell cortex is not saturable, as expected for a lipid-binding protein. Importantly, the localization of GFP-Blt1 to the cell periphery was also independent of Cdr2, which recruits endogenously expressed Blt1 to medial cortical nodes (see Fig. S5E) (11). These results suggest that Blt1 may interact with the lipid bilayer. Despite its abundance at the cell cortex, GFP-Blt1 was excluded from the cortex at growing cell ends, as marked by the cell wall dye blankophor (see Fig. S5C). In addition, GFP-Blt1 localized to the nongrowing end of monopolar mutants, indicating that its exclusion at cell ends is not due to membrane curvature or geometry (see Fig. S5E). Rather, this was reminiscent of the previous studies that have shown the exclusion of other proteins at sites of cell growth (9, 11–13), perhaps due to a different lipid composition and/or membrane flux at these sites.

Domain analysis of Blt1. The only sequence-predicted domain in Blt1 was a leucine zipper motif between residues 488 to 575. To search for additional functional domains, we generated a panel of Blt1 truncation mutants that were expressed from multicopy plasmids under the control of the medium-strength P41nmt1 promoter in *blt1*Δ cells. In the repressed state, full-length Blt1 expressed by this promoter localized similarly to the endogenous protein. When induced, this promoter drove overexpression of Blt1, leading to localization throughout the nongrowing cell cortex (Fig. 3). We used this expression system to determine the localization of 12 truncation mutants under endogenous and overexpression conditions (Fig. 3; see also Fig. S6 in the supplemental material).

Upon overexpression, we observed a strong localization at the cell periphery for all constructs containing a C-terminal domain that encompassed residues 575 to 700 (e.g., *blt1*Δ*1* and *blt1*Δ*4* [Fig. 3; see also Fig. S6 in the supplemental material]). In contrast, constructs lacking this C-terminal domain (e.g., *blt1*Δ*5*) were not found at the cell periphery upon overexpression. This means that a C-terminal 125-amino-acid domain is both required and sufficient for Blt1 association with the cell periphery. Interestingly, Blt1 localization to medial cortical nodes at endogenous expression levels appears to be independent of the C terminus. For example, the *blt1*Δ*1* mutant that lacks the N-terminal 79 residues of Blt1 was not concentrated at medial cortical nodes at endogenous expression levels, despite its strong localization to the cell periphery upon overexpression. Moreover, two constructs (*blt1*Δ*5* and *blt1*Δ*6*) lacking the C-terminal domain maintained localization to medial cortical nodes when expressed at endogenous levels, whereas we observed cytoplasmic localization for the *blt1*Δ*8* mutant, which truncates both the N- and C-terminal domains (Fig. 3; see also Fig. S6). We concluded that the N terminus of Blt1 gov-

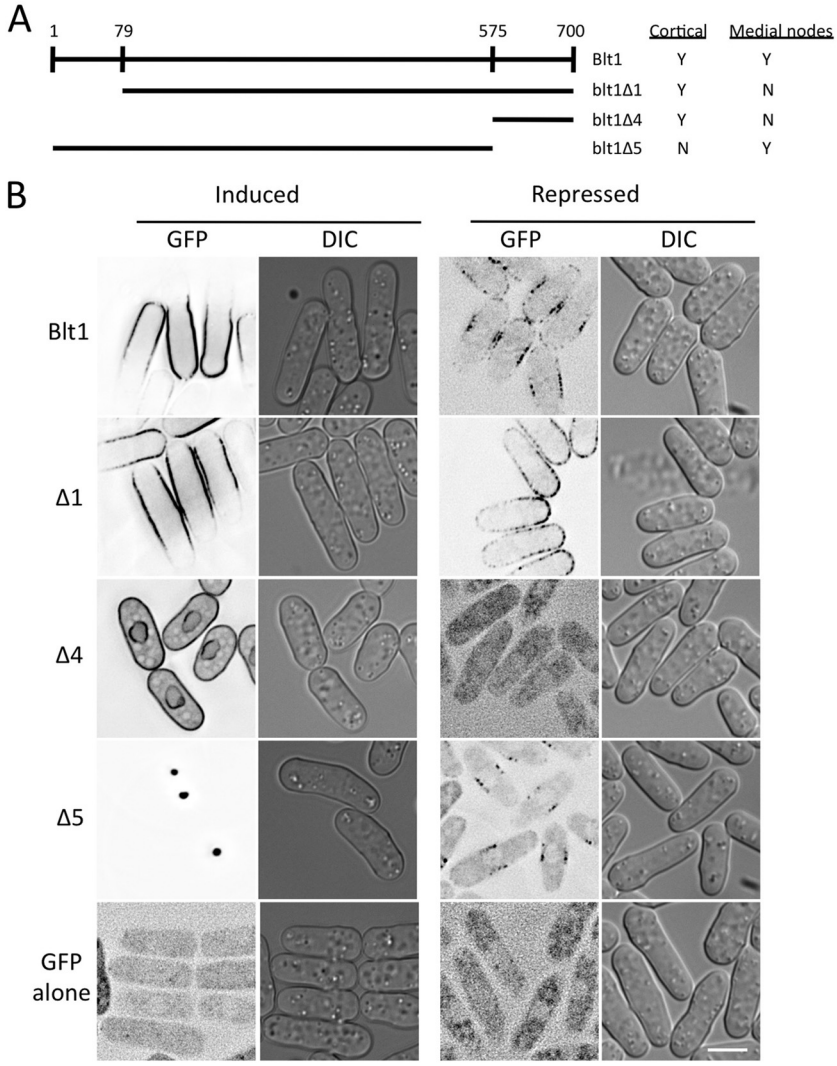

FIG 3 Domain analysis of Blt1. (A) Schematic of key truncation constructs. Right columns indicate whether the constructs localize to the cell cortex upon overexpression or to medial nodes upon low-level expression. Y, yes; N, no. A complete list of constructs and localization data are provided in Fig. S6 of the supplemental material. (B) Images of the Blt1 truncation constructs from panel A, expressed as GFP-fusion proteins in *blt1*Δ cells (strain JM429). Images are inverted single deconvolved focal planes with the accompanying DIC image. Overexpression was induced by removal of thiamine for 24 h; expression under repressed conditions was similar to endogenous Blt1 levels. Bar, 5 μm.

erns localization to medial cortical nodes, while a separable C-terminal domain may drive association with membrane lipids.

The Blt1 N terminus directs node localization and function. To further investigate the role of the Blt1 N terminus, we integrated the *blt1Δ1* truncation mutant with an mCherry tag under the control of the endogenous promoter as the sole copy in the genome. *blt1*Δ1-mCherry localized to the cell cortex asymetrically but did not colocalize with Cdr2-mEGFP in cortical nodes (Fig. 4A). This sharply contrasted with the colocalization of wild-type Blt1-mCherry with Cdr2-mEGFP and mimicked the localization defect observed for Blt1 in *cdr2*Δ cells. Indeed, the localization of Blt1-mCherry and blt1Δ1-mCherry was identical in *cdr2*Δ cells (Fig. 4B), indicating that this N-terminal domain is required for Blt1 recruitment to medial cortical nodes by Cdr2. In support of this conclusion, the physical interaction of Cdr2 and Blt1 by coimmunoprecipitation was reduced in the *blt1Δ1* mutant (see Fig. S7 in the supplemental material).

We next tested the function of the Blt1 N terminus in cortical recruitment of Mid1-Nter for cytokinesis. Cortical *blt1Δ1* failed to recruit Mid1-Nter to cortical nodes, in contrast to full-length Blt1 (Fig. 4C). Consistent with this defect in Mid1-Nter localization, we found that Gef2-mEGFP was absent from interphase cortical nodes of *blt1Δ1* cells and did not colocalize with blt1Δ1-mCherry at the cell cortex (see Fig. S7B in the supplemental material). This suggests that the N terminus of Blt1 is required for recruitment of Gef2 to interphase cortical nodes, and Gef2 then acts as an adaptor for Mid1-Nter. We note that Gef2 localized to the cytokinetic ring in *blt1Δ1* cells (see Fig. S7C), similar to *blt1*Δ cells (31), indicating an independent recruitment mechanism later in the cell cycle. The failure in recruitment of Gef2 and Mid1-Nter to medial cortical nodes led to functional defects, as the *blt1Δ1* mutation exhibited synthetic defects with *mid1-Nter* identical to *blt1*Δ (Fig. 4D). We concluded that the N terminus of Blt1 is required for Blt1 recruitment to cortical nodes by Cdr2 and subsequent recruitment of Gef2 and Mid1-Nter to the cell cortex.

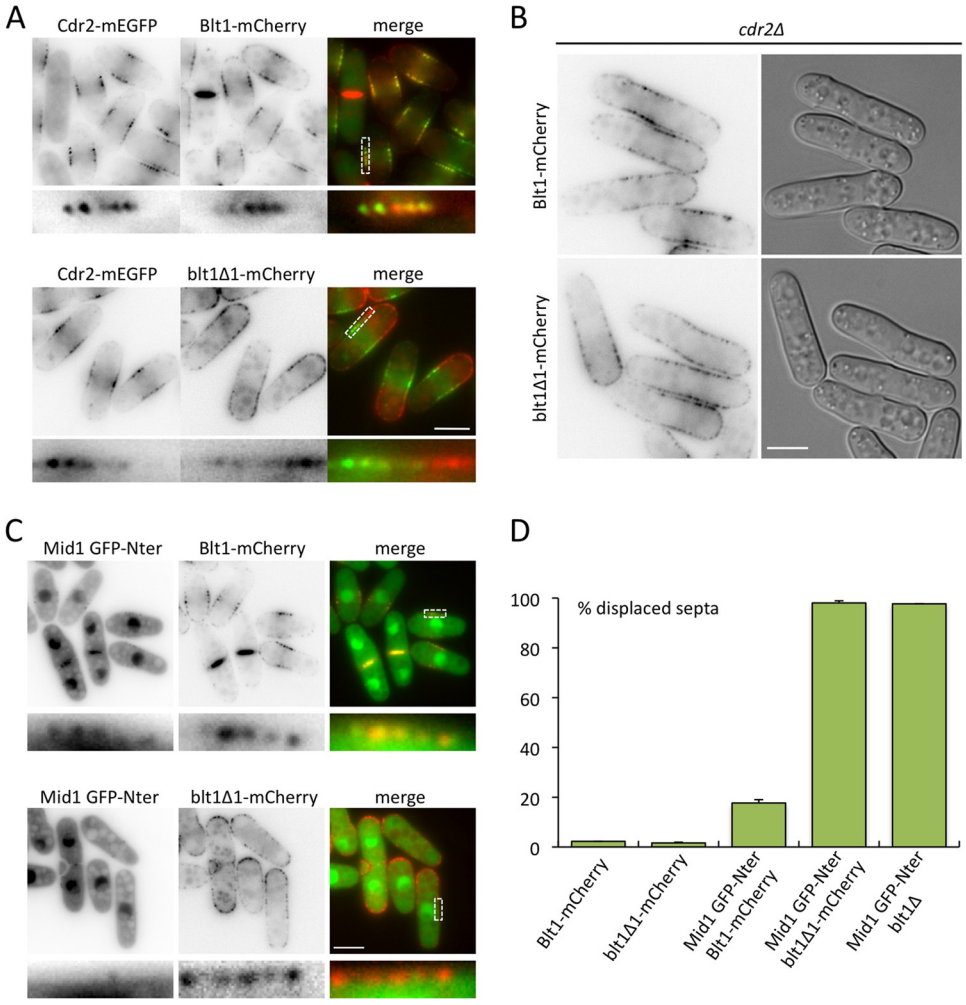

FIG 4 The N terminus of Blt1 is required for localization and function. (A) Cdr2-mEGFP colocalizes with Blt1-mCherry but not blt1Δ1-mCherry. Images are inverted single focal planes; bottom rows are magnified views of the white, boxed regions. Bar, 5 μm. Strains used were JM2193 and JM2194. (B) Localization of Blt1-mCherry and blt1Δ1-mCherry in *cdr2*Δ cells. Left images are inverted single focal planes; right images were produced by DIC. Bar, 5 μm. Strains used were JM2197 and JM2198. (C) Mid1 GFP-Nter colocalizes with cortical Blt1-mCherry but not blt1Δ1-mCherry. Images are presented as described for panel A. Bar, 5 μm. Strains used were JM1681 and JM1682. (D) Percentages of displaced septa for the indicated genotypes (strains JM1684, JM1685, JM1681, JM1682, and JM1680). Bars represent means ± standard deviations (error bars) from two separate experiments ($n > 200$ cells in each experiment).

The Blt1 C terminus associates with lipids to direct cortical localization. Our data suggest that Blt1 is recruited to cortical nodes by an N-terminal domain that associates with Cdr2, but it can independently target the cell cortex through a C-terminal domain that may interact with membrane lipids. To analyze the function of this Blt1 C-terminal domain in more detail, we integrated the *blt1*Δ*5* mutant, which lacks the C-terminal domain, with a GFP tag under the control of the endogenous promoter as the sole copy in the genome. Consistent with our plasmid-based results, *blt1*Δ*5*-mEGFP localized to medial cortical nodes despite lacking the membrane-binding domain (Fig. 5). However, this localization of *blt1*Δ*5*-mEGFP to the cell cortex was abolished in *cdr2*Δ cells (Fig. 5). This contrasted with full-length Blt1 and the *blt1*Δ*1* mutant, which both remained cortical in *cdr2*Δ cells (Fig. 4B). These data confirmed that Blt1 has separable domains to target medial cortical nodes versus the cortex in general.

We next tested the possibility that the Blt1 C terminus targets the cell cortex by binding to lipids. Cell extracts containing either full-length GFP-Blt1 or the truncated GFP-blt1Δ5 (see Fig. S8 in the supplemental material) were incubated with lipid array strips. Using anti-GFP antibodies, we found that Blt1 interacted with negatively charged phospholipids but *blt1*Δ*5* did not (Fig. 6A). This indicated that Blt1 is a membrane-binding protein, although we note that this interaction could be indirect. In support of a direct interaction with lipids, sequence analysis of the C-terminal domain revealed several clusters of positively charged residues that could facilitate association with negatively charged lipids in cellular membranes (Fig. 6B). Truncation of the C-terminal 26 residues, including two adjacent lysines, did not impair cortical localization. In contrast, truncation to delete additional clusters of basic residues abolished cortical localization (Fig. 6B and C). These data strongly suggest the possibility that basic residues in the C-terminal domain establish electrostatic interactions with negatively charged phospholipids to promote Btl1 cortical localization in cells.

Cortical targeting by the Blt1 C terminus is important for cytokinesis. Finally, we tested the role of the Blt1 C-terminal do-

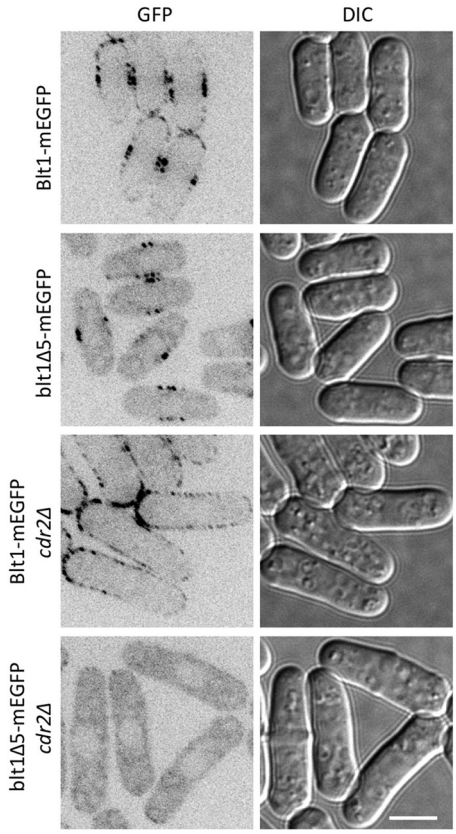

FIG 5 Cdr2 is required for cortical localization of blt1Δ5 but not full-length Blt1. Localization of endogenous Blt1 and truncated blt1Δ5 in wild-type and *cdr2*Δ cells. Images are inverted single confocal planes. Bar, 5 μm. Strains used were JM151, JM216, JM1607, and JM1642.

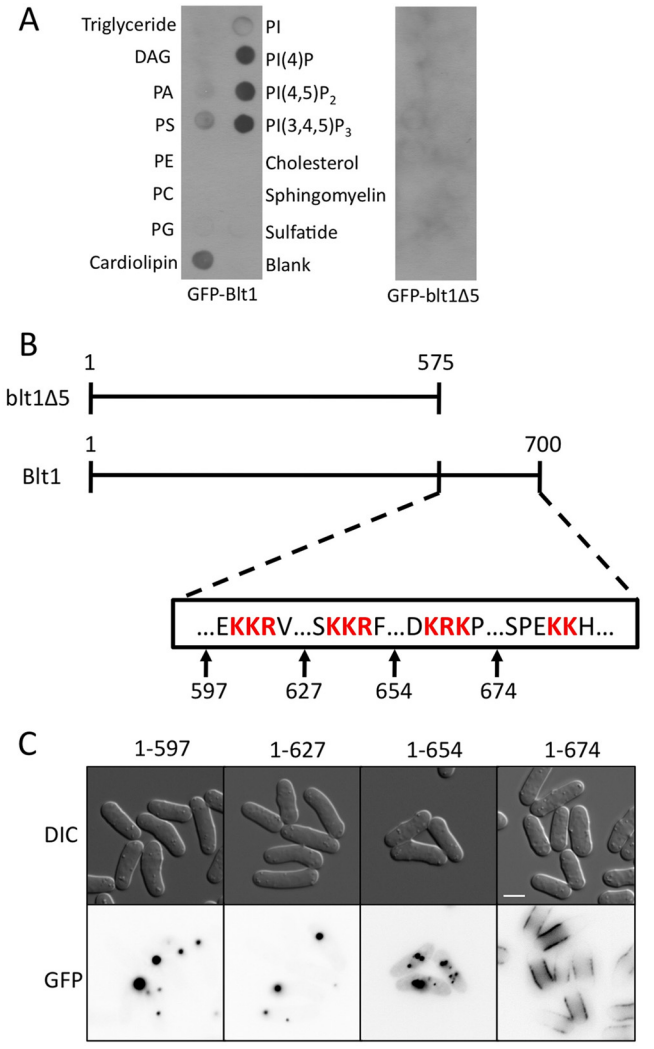

FIG 6 Blt1 interacts with lipids through a C-terminal domain. (A) Whole-cell extracts from cells overexpressing GFP-Blt1 or the truncated GFP-blt1Δ5 in strain JM429 were incubated with lipid strip arrays and analyzed by Western blotting using anti-GFP antibodies. (B) Schematic of truncated blt1Δ5 and full-length Blt1. Clusters of basic residues are highlighted. (C) Localization of the indicated constructs upon overexpression in *blt1*Δ cells. Bar, 5 μm. The strain used was JM429.

main in cytokinesis. Interestingly, we found that *blt1Δ5* recruited and colocalized with Mid1-Nter at medial cortical nodes during interphase (Fig. 7A). Thus, deleting the membrane-binding domains of both Mid1 and Blt1 did not prevent their coaccumulation at medial cortical nodes. This indicated that Blt1 acts as a scaffold, independent of its membrane-binding activity, to promote the robust localization of Mid1 at medial cortical nodes.

This result raised the possibility that the Blt1 C terminus is dispensable for function, because the Blt1 N terminus mediates localization to cortical nodes and downstream recruitment of Mid1-Nter. However, the *blt1Δ5* and *mid1-Nter* mutations exhibited strong synthetic defects in positioning the division plane (Fig. 7B and C). Truncating the C-terminal membrane-binding domain of either Mid1 or Blt1 did not lead to septation defects, but nearly all *blt1Δ5 mid1-Nter* double mutants displayed misplaced septa.

To determine the underlying mechanism that leads to septation defects in *blt1Δ5 mid1-Nter* cells, we used time-lapse microscopy of cells expressing Blt1-mEGFP or *blt1Δ5*-mEGFP. These cells also expressed the actomyosin ring marker Rlc1-mCherry and the spindle-pole body (SPB) marker Sfi1-mCherry. We observed the expected pattern of SPB separation and actomyosin ring assembly in *blt1Δ5* cells and only minor defects in *mid1-Nter* cells (Fig. 8A). In contrast, the double mutant displayed synthetic defects in spatial control of actomyosin ring assembly (Fig. 8A). Moreover, we observed that *blt1Δ5*-mEGFP rapidly detached from the cortex upon SPB separation, unlike full-length Blt1-mEGFP. The mutant *blt1Δ5*-mEGFP then reappeared as a large clump containing both *blt1Δ5*-mEGFP and Rlc1-mCherry (Fig. 8A, arrow heads). Mid1-Nter displayed similar behavior in early mitotic cells (see Fig. S9 [arrow heads] in the supplemental material). An actomyosin structure containing both Rlc1-mCherry and *blt1Δ5*-mEGFP assembled abnormally from these clumps, often resulting in elongated actomyosin filaments that reached the cell tips, reminiscent of the *mid1Δ* phenotype. These cytokinetic filaments at cell tips were only observed in the *blt1Δ5 mid1-Nter* double mutants (Fig. 8C). These results suggest that nodes containing *blt1Δ5* and Mid1-Nter are destabilized at mitotic entry in the absence of membrane binding by both Mid1 and Blt1. This lack of stability leads to defective recruitment of cytokinesis proteins, including myosin II, resulting in misplaced cytokinetic rings and septa.

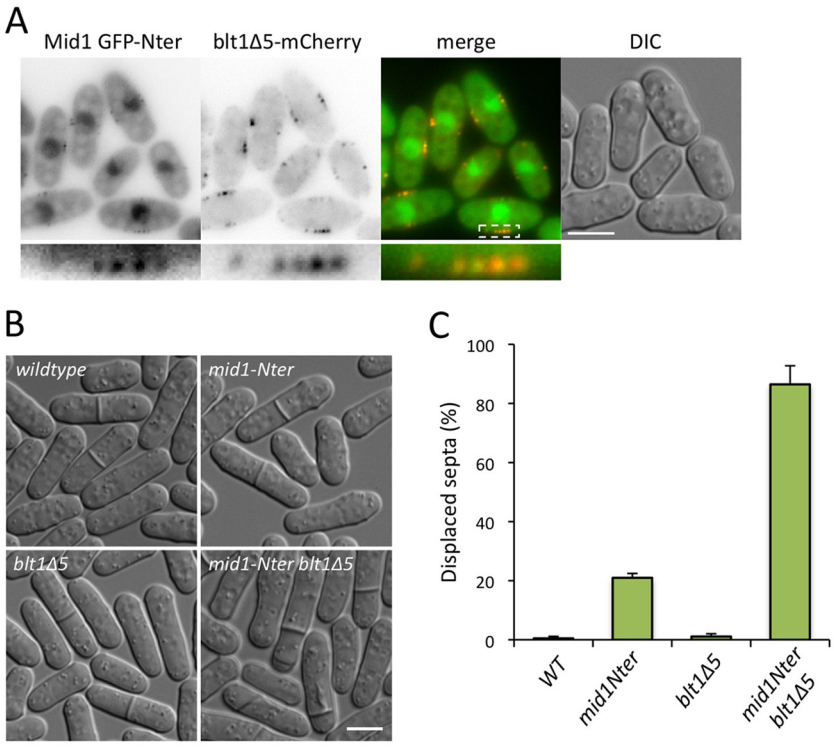

FIG 7 The Blt1 membrane-binding domain is required for Mid1-Nter function. (A) Mid1 GFP-Nter and Blt1Δ5-mCherry colocalize in cortical nodes. Images are inverted single deconvolved focal planes. The lower row is a magnified view of the boxed region. Bar, 5 μm. The strain used was JM1603 (B) DIC images of wild-type, *mid1-Nter*, *blt1Δ5-mCherry*, and *mid1-Nter blt1Δ5-mCherry* cells (strains JM1598, JM1603, JM1595, and JM1597). Bar, 5 μm. (C) Percentages of displaced septa in the indicated genotypes. Bars represent means ± standard deviations (error bars) from two separate experiments ($n > 200$ cells in each experiment). The strains used are the same as for panel B.

Why would *blt1Δ5 mid1-Nter* nodes dissociate from the cortex at mitotic entry? We considered a role for Cdr2, which recruits the *blt1Δ5* mutant to cortical nodes but dissociates from nodes at mitotic entry (10). During this stage, nodes mature by the recruitment of many cytokinesis proteins and then compact into a cytokinetic ring (5). If Cdr2 dissociation from nodes leads to detachment of *blt1Δ5* and Mid1-Nter, then constitutive tethering of Cdr2 to the cell cortex might suppress this defect. To test this prediction, we artificially tethered Cdr2 to the cell membrane by fusing the membrane-binding C-terminal fragment of Mod5 (containing basic residues and a CAAX prenylation motif) to the C terminus of Cdr2-GFP. Unlike wild-type Cdr2-GFP, this Cdr2-GFP-CAAX fusion protein was present at the cell cortex throughout cytokinesis (see Fig. S10 in the supplemental material). Consistent with our prediction, the addition of Cdr2-CAAX partially suppressed the septum-positioning defect of *blt1Δ5 mid1-Nter* double mutant cells (Fig. 9). We concluded that Cdr2 serves as the sole cortical anchor for cytokinetic nodes in these double mutant cells, and dissociation of Cdr2 from nodes during ring assembly leads to node detachment and cytokinesis defects.

DISCUSSION

Assembly of the contractile ring in fission yeast begins with the positioning of cortical nodes in the cell middle (1, 3, 5), followed by maturation and condensation of nodes into a contractile ring during mitosis. A similar process assembles the contractile ring in animal cells, where cytokinesis proteins are recruited to a broad equatorial band that subsequently condenses to form the mature actomyosin ring (32, 33). Our work provides molecular insight into the mechanisms that assemble and anchor cytokinetic nodes at the plasma membrane in fission yeast, with implications for cytokinetic ring assembly in a broad range of cell types.

We found that Blt1 acts as both a scaffold and a cortical anchor for the anillin-like protein Mid1, with Blt1-Mid1 interactions bridged by Gef2. The N terminus of Blt1 is required for localization to cortical nodes and for the recruitment of Gef2 and Mid1-Nter. It is currently unknown if Blt1 and Gef2 bind directly or indirectly, but we note that the C terminus of Gef2 is crucial for Gef2 interaction with nodes (31), raising the possibility that the Blt1 N terminus interacts with the Gef2 C terminus. We also note that additional uncharacterized proteins may facilitate these functional interactions. Scaffold proteins are emerging as key integrators of multicomponent systems, such as medial cortical nodes (34). In this scaffolding function, Blt1 may ensure robust assembly of the cell division machinery by integrating spatial and temporal information with physical connections to the cell cortex.

Blt1 scaffolding activity is independent from its interactions with the membrane, which depend on the C terminus. Several lines of evidence suggest that Blt1 may directly bind to lipids: (i) nonsaturable binding of Blt1 to the cortex upon overexpression; (ii) *in vitro* binding of Blt1-GFP but not truncated *blt1Δ5*-GFP to lipids in cell extracts; (iii) the presence of C-terminal basic-rich motifs with the potential to mediate the electrostatic interaction with acidic phospholipids, such as phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], enriched at the plasma membrane. Syn-

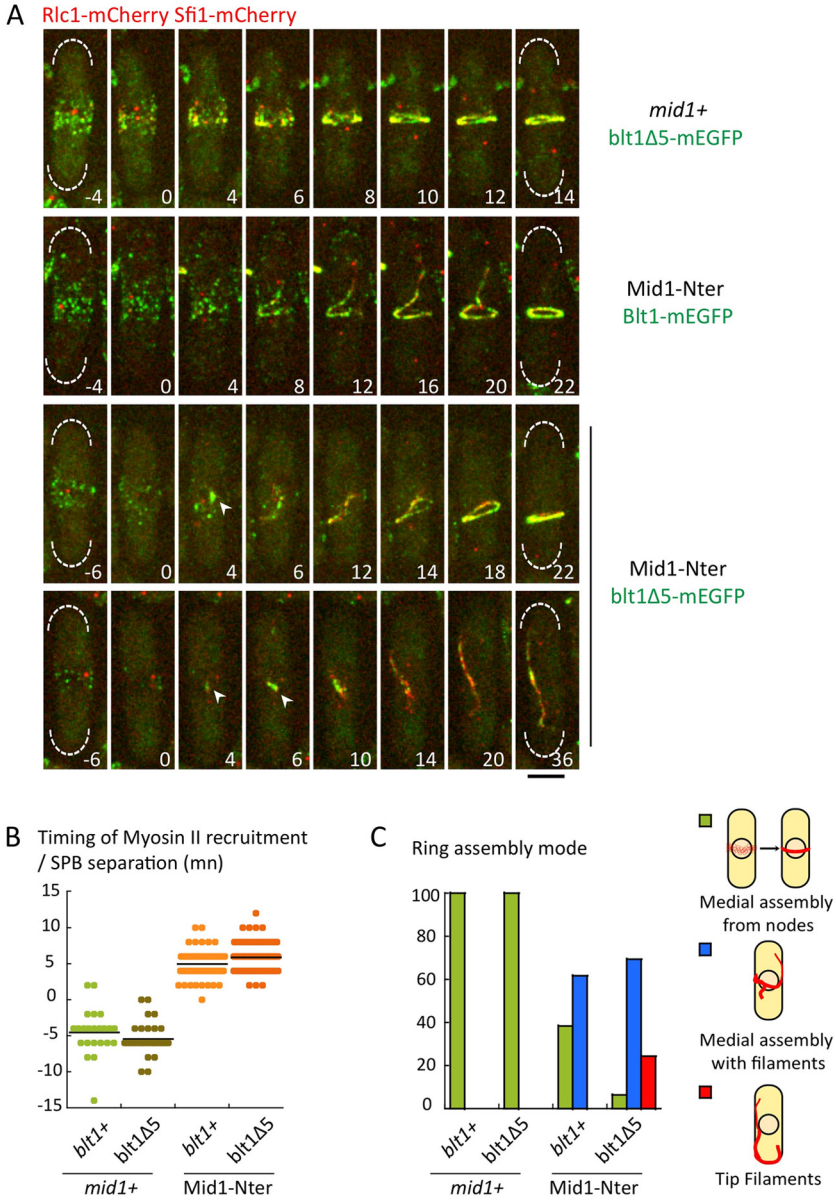

FIG 8 Cortical nodes disassemble during mitosis in *mid1-Nter blt1*Δ*5* cells. (A) Live cell imaging of the *mid1-Nter blt1*Δ*5-mEGFP* strain expressing the myosin II light chain Rlc1-mCherry and the SPB marker Sfi1-mRFP as a timer of mitotic entry (AP3925). mid1-Nter Blt1-mEGFP (AP3924) and *mid1*$^{+}$ *blt1*Δ*5-mEGFP* (AP3907) control strains are also shown. Note that at mitosis entry in the Blt1Δ5-mEGFP Mid1Nter mutant, Blt1Δ5-mEGFP nodes disappeared from the cortex, while large clusters containing both Blt1Δ5-mEGFP and Rlc1-mCherry assembled (arrow heads). The Rlc1-mCherry recruitment pattern was strongly affected. Images are maximum projections from spinning disc *z*-series. Bar, 5 μm. (B and C) Quantification of the timing of Rlc1-mCherry recruitment to nodes (B) and ring assembly mode (C). Measurements were performed using the same strains as for panel A and also a wild-type strain expressing the same markers (AP3906). Bars represent means ± standard deviations (error bars) from two separate experiments ($n > 200$ cells in each experiment).

thetic defects between the *blt1*Δ*5* and *mid1-Nter* mutants indicated that Blt1 interactions with the membrane act in parallel to the Mid1 membrane-binding helix.

Based on these results, we propose a model for the interactions between Mid1, Blt1, Gef2, Cdr2, and the plasma membrane within a node (Fig. 10). When membrane anchoring by both Mid1 and Blt1 is impaired, we propose that nodes remain cortical during interphase due to Cdr2, which may also facilitate interactions among Blt1, Gef2, and Mid1. However, Cdr2 leaves the cortex during mitosis, leading to node detachment and disassembly. Without proper cortical node attachment to the plasma membrane, the cytokinetic ring assembles from elongated clumps to generate severe cytokinesis defects. The formation of a clump-derived actomyosin filament in these cells resembles aberrant cytokinesis in *mid1*Δ mutants (35, 36). In support of our model, a Cdr2 construct artificially tethered to membranes during mitosis (Cdr2-GFP-CAAX) partially rescued the division plane definition defects of the *mid1-Nter blt1*Δ*5* double mutant. We note that the broader distribution of Cdr2-CAAX on the cortex compared to Cdr2 (see Fig. S10 in the supplemental material) may preclude a complete rescue.

Our model accounts for the phenotypes that we have de-

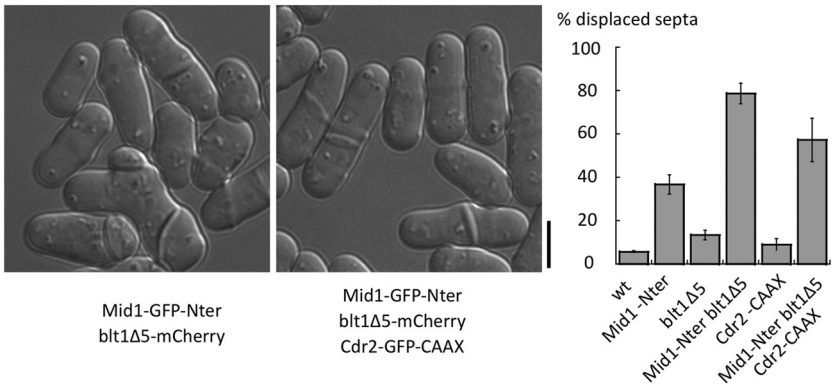

FIG 9 Cdr2-GFP-CAAX partially rescues septum-positioning defects in the *mid1-Nter blt1Δ5* mutant. DIC images (left) and percentages of displaced septa (right) in *Mid1-GFP-Nter* (AP998), *blt1Δ5-mCherry* (JM1597), *Mid1-GFP-Nter blt1Δ5-mCherry* (JM1603), *Cdr2-GFP-CAAX* (AP3898), and *Mid1-GFP-Nter Blt1Δ5-mCherry Cdr2-GFP-CAAX* (AP3909) strains are shown. Bars represent means ± standard deviations (error bars) from three separate experiments (n = 300 cells in each experiment).

scribed, but more complex relationships are likely to exist in cells. For example, in *mid1*(Δ300-350) and *gef2Δ* mutants, Blt1 is less concentrated in medial nodes (see Fig. S3 in the supplemental material), indicating bidirectional regulation or feedback in the assembly of these structures. These nodes also contain additional proteins, including the kinesin-like protein Klp8 and the membrane-binding F-BAR protein Cdc15, with physical links to the Blt1-Mid1-Gef2-Cdr2 module (11). Further, multiple copies of these proteins are present in each node, and oligomerization and multivalent interactions are likely to generate more complex, higher-order structures. We anticipate that additional proteins and interactions will be uncovered in future work on these cortical node structures.

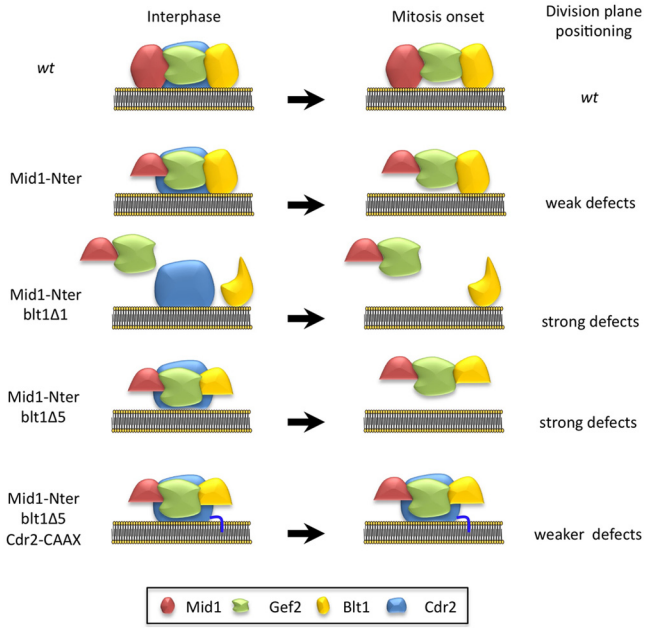

FIG 10 Model of proposed molecular interactions in medial cortical nodes. During interphase, Cdr2 recruits Mid1 and Blt1 to medial cortical nodes. Gef2 reinforces the stability of the complex by mediating additional Mid1-Blt1 indirect interactions. Cortical anchoring of the complex is ensured in a redundant manner by Cdr2, Blt1, and Mid1. The Blt1 N terminus, deleted in the *blt1Δ1* mutant, controls Blt1 recruitment to cortical nodes and subsequent recruitment of Gef2 and Mid1-Nter, which is deficient for membrane binding. The Blt1 C terminus, deleted in *blt1Δ5*, acts as a membrane anchor for Blt1. The interactions of Mid1 and Blt1 with membranes are dispensable for recruitment of Mid1-Gef2-Blt1 to nodes during interphase due to association with Cdr2. However, Cdr2 dissociation from the cortex during mitosis creates a requirement for membrane binding by either Mid1 or Blt1. At this stage, cortical nodes in the double mutant *mid1-Nter blt1Δ5* dissociate from the cortex, leading to division plane-positioning defects. Artificial node anchoring during mitosis by Cdr2-CAAX can partially suppress these defects. Note that protein interactions in the model may be indirect and involve additional factors.

We note that Blt1 is necessary for Gef2 association with medial cortical nodes during interphase (11), but Gef2 localizes to the cytokinetic ring independently of Blt1 during mitosis (31). Accordingly, we found that Gef2 localized to the contractile ring in *blt1Δ1* cells but not with medial cortical nodes during interphase (see Fig. S7C in the supplemental material). In contrast, *gef2Δ* cells display a lack of Mid1-Nter at the contractile ring (31) and decreased levels of Blt1 at the contractile ring (see Fig. S11 in the supplemental material). These results suggest that Gef2 may be independently recruited to the assembled contractile ring by other factors to promote Mid1-Nter and Blt1 recruitment. Further work is necessary to resolve the underlying molecular mechanisms.

The overlapping roles of the Blt1 and Mid1 membrane-binding domains highlight the importance of stable attachment of structures such as nodes to the plasma membrane. During contractile ring assembly, medial cortical nodes mature by sequentially recruiting components of the contractile ring, while Cdr2 dissociates from nodes. Cortical nodes become mobile within the cell cortex at this stage, driving assembly of the mature cytokinetic ring (4, 5). We found that interactions of Blt1 and Mid1 with the membrane are crucial in this time frame. This suggests that multiple connections to membrane lipids may stabilize node attachment to the plasma membrane during dynamic node movements.

Animal cells undergo a similar process, where cytokinesis factors, including the Mid1-like protein anillin, are initially recruited to a broad equatorial band that subsequently condenses into a compact ring (32, 33). As in fission yeast, overlapping membrane anchors may be required to ensure movement of these multiprotein assemblies within the plane of the cell cortex. It is interesting that Blt1 associates with negatively charged phospholipids, including $PI(4,5)P_2$, which recruits the Mid1-like protein anillin to the central cortex during animal cell cytokinesis (37). This sug-

gests that similar interactions between proteins and lipids may underlie the robust assembly of the cytokinetic ring in diverse cell types and organisms.

A separate role for membrane-binding domains may be the spatial organization of phospholipids in the membrane. The Blt1 membrane-binding domain associates with negatively charged phospholipids, such as $PI(4,5)P_2$, which acts as a signaling molecule during cytokinesis (38). Thus, membrane-binding domains may also promote signaling pathways through the recruitment and/or retention of specific lipids. A growing number of protein-protein and protein-lipid interactions are being uncovered in the cytokinetic ring (2, 4, 5). By defining the physical interactions within cytokinetic ring precursors, such as cortical nodes, we anticipate the discovery of additional mechanisms that promote proper positioning and assembly of the cell division machinery. These interactions between protein and lipid components of the cytokinetic ring drive the dynamic events that ensure faithful cycles of cell division.

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Supplementary Figure Legends

Table S1. Strains used in this study

Figure S1. Co-localization of Mid1 GFP-Nter and Blt1-mCherry in medial cortical nodes and the cytokinetic ring. Images are inverted single focal planes. Strain is AP2165. Scale bar, 5 μm.

Figure S2. Mid1 residues 300-350 control Mid1-Nter localization to the contractile ring. (A) Localization of Mid1 GFP-Nter in cells containing Rlc1-mCherry rings or strands. Scale bar: 5μm. Images are maximum projections of spinning disc confocal z-series; same cells are shown in top and bottom rows. Strains AP3900, AP3902. (B) Percentage of cells containing Rlc1-mCherry rings or strands positive for Mid1 GFP-Nter (n=95) or Mid1 GFP-NterΔ300-350 (n=64). (C) Localization of Mid1 GFP-(300-350) in cells containing Rlc1-mCherry rings or strands in wild type or *blt1*Δ backgrounds. Scale bar: 5μm. Images are maximum projections of spinning disc confocal z-series; same cells are shown in top and bottom rows. Strains AP3901, AP3910. (D) Percentage of cells containing Rlc1-mCherry rings or strands positive for Mid1 GFP(300-350) in wild type (n= 65) or *blt1*Δ backgrounds (n=69).

Figure S3. Localization of Blt1-mEGFP in (A) *mid1*+ or (B) *mid1*Δ*300-350* cells during interphase. Images are inverted single focal planes. Scale bar, 5 μm. Right, line scans of fluorescence intensity along the left side cortex of individual cells, starting at the bottom cell tip.

Note that the clear peak of Blt1-mEGFP at the cell middle is lost in *mid1*Δ*300-350* cells. Strains AP3490, AP3491.

Figure S4. Mid1(300-450) interacts with Blt1 through Gef2. (A) Co-immunoprecipitation assays between Blt1-mEGFP and GST-Mid1(300-450). Immunoprecipitation was performed with an anti-GFP mAb, or with an anti-HA mAb as negative controls. Input and IP samples were probed with anti-Mid1 antibodies. (B) Co-immunoprecipitation assays between Blt1-mEGFP and GST-Mid1(300-450) in wild type (*gef2*+) or *gef2*Δ mutant cells. Experiments were performed as in panel A, and normalized signal quantification from two independent experiments is shown on the right. (C) Co-immunoprecipitation assays between GST-Mid1(300-450) and Gef2-mEGFP in wild-type and *blt1*Δ cells. Immunoprecipitation was performed as in A. Normalized signal quantification from two independent experiments is shown on the right. Strains used were AP3645, AP3866, AP3867, AP3868.

Figure S5. Blt1 localizes to the cell cortex. (A) Left: localization of Blt1-mEGFP and Rlc1-mRFP in the cytokinetic ring. Right: localization of Blt1-mCherry and Rlc1-mEGFP in the cytokinetic ring. Image reconstructed from a deconvolved Z-series, strains are JM191 and JM193. Scale bar, 1 μm. (B) Left: localization of Blt1-mEGFP and Rlc1-mRFP in the cytokinetic ring; images are a single focal plane from a deconvolved Z-series. Right: Quantification of fluorescence intensity for Blt1-mEGFP (green) and Rlc1-mRFP (red) from left panel images. Note that peak intensity of red signal is inside peak intensity of green signal. Strain is JM191. Scale bar, 1 μm. (C) Left, localization of over-expressed GFP-Blt1. Right, blankophor staining to mark growing cell ends and division septa. Images are inverted maximum projections

from deconvolved Z-series; strain is JM284. Scale bar, 3 μm. (D) Deconvolved Z-series of GFP-Blt1 over-expressing cells. Scale bar, 5 μm. (E) Localization of over-expressed GFP-Blt1 in the indicated mutants. *pom1*Δ, *tea1*Δ, and *ppk2*Δ cells exhibit monopolar growth. Top row, inverted single deconvolved focal plane. Bottom row, Blankophor staining to mark growing cell ends. Strains are JM771, JM772, JM773, and JM776. Scale bar, 5 μm.

Figure S6. Domain analysis of Blt1. (A) Schematic of truncation constructs. Right columns indicate whether the constructs localize to the cell cortex upon over-expression, and to medial nodes upon low-level expression. Y, yes; N, no. (B) Images of the Blt1 truncation constructs from panel A expressed as GFP fusion proteins in *blt1*Δ cells (strain JM429). Images are inverted single deconvolved focal planes with accompanying DIC image. Over-expression was induced by removal of thiamine for 24 hours; expression under repressed conditions is similar to endogenous Blt1 levels. Scale bar, 5 μm. Note that constructs from Figure 3 are shown in duplicate in this supplementary figure to present the complete domain analysis.

Figure S7. Defects of the blt1Δ1 mutant. (A) blt1Δ1 exhibits reduced interactions with Cdr2. Co-immunoprecipitation assays between Cdr2-3HA and GFP-Blt1, GFP-blt1Δ1, or GFP alone. Immunoprecipitation was performed with an anti-GFP Ab, input and IP samples were probed with anti-HA antibodies. GFP proteins were over-expressed from plasmids in strain JM1652. The quantification (right panel) shows a ratio of signal for IP versus input in each sample normalized to full-length GFP-Blt1. (B) Gef2 is absent from the cell cortex in blt1Δ1 cells. Co-localization of Gef2 with either Blt1-mCherry or blt1Δ1-mCherry. Images are inverted single

focal planes; bottom rows are magnified views of white, boxed regions. Scale bar, 5 μm. Strains are JM2195 and JM2196. (C) Gef2-mEGFP localizes to cytokinetic rings in *blt1*Δ*1* cells.

Figure S8. Solubility of over-expressed GFP-Blt1 and GFP-blt1Δ5. Soluble supernatants were used to probe Blt1 interactions with lipid strip arrays in Figure 6. Equal amounts of whole-cell extract (WCE) or clarified supernatant (supe) were separated by SDS-PAGE and analyzed by Western blot using anti-GFP antibodies. GFP-Blt1 and GFP-blt1Δ5 were over-expressed on pREP41 plasmids in strain JM429.

Figure S9: Formation of aberrant clumps in *mid1Nter blt1*Δ*5* nodes cells during early mitosis. (A) Localization of GFP-Mid1Nter and Blt1-mCherry (AP2165) or GFP-Mid1Nter and Blt1Δ5-mCherry (JM1603) in early mitotic cells. Note that when Blt1 membrane-anchoring domain is deleted, Mid1Nter and Blt1 form large clumps (arrowheads) in early mitosis while medial nodes largely disappear. Clumps were observed in 43/45 *mid1-Nter blt1*Δ*5* cells, compared to 0/33 *mid1-Nter blt1*+ cells. Max projections of spinning disc Z-series. Scale bar: 5μm.

Figure S10: **Cdr2-GFP-CAAX mutant does not detach from the cortex during mitosis.** Timelapse movie of Cdr2-GFP and Cdr2-GFP-CAAX mutant. Spinning disc confocal single focal plane. Scale bar: 5 μm. Strains AP3177, AP3898. Note that Cdr2-GFP-CAAX does not detach from the cortex during mitosis, in contrast to Cdr2-GFP**.**

Figure S11. Localization of Blt1-mEGFP in wild type versus *gef2*Δ cells during mitosis and septation. Images are inverted single focal planes. Note lower reduced concentration of Blt1-mEGFP in *gef2*Δ cells, as compared to wildtype cells. Scale bar, 5 μm.

Strain	Genotype	Source
JM151	*blt1-mEGFP::kanMX6 ade6-M21X leu1-32 ura4-D18 h-*	(3)
JM191	*rlc1-mRFP::nat blt1-mEGFP::kanMX6 ura4-D18 leu1-32 ade6-M21X h+*	This study
JM193	*rlc1-GFP::kanMX6 blt1-mCherry::nat ura4-D18 leu1-32 ade6-M21X h-*	This study
JM216	*blt1-mEGFP::kanMX6 cdr2Δ::ura4+ ura4-D18*	(3)
JM251	*blt1Δ::natR h+*	(3)
JM284	*kanMX6-P3nmt1-GFP-blt1 ura4-D18 leu1-32 ade6-M21X h+*	This study
JM365	*gef2-mEGFP::kanMX6 ura4-D18 leu1-32 ade6-M21X h-*	(3)
JM366	*972 h-*	lab collection
JM429	*blt1Δ::kanMX6 ade6-M216 leu1-32 ura4-D18 h+*	(3)
JM771	*kanMX6-P3nmt1-GFP-blt1 cdr2Δ::natR ura4-D18 leu1-32 ade6-M21X*	This study
JM772	*kanMX6-P3nmt1-GFP-blt1 pom1Δ::ura4+ ura4-D18 leu1-32 ade6-M21X*	This study
JM773	*kanMX6-P3nmt1-GFP-blt1 tea1Δ::ura4+ ura4-D18 leu1-32 ade6-M21X*	This study
JM776	*kanMX6-P3nmt1-GFP-blt1 ppk2Δ::ura4+ ura4-D18 leu1-32*	This study
JM1595	*blt1-mCherry::natR mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- + pSM26 integrated (pmid GFP-Nter mid1 1-506, leu1+)*	This study
JM1597	*blt1(1-575)-mCherry::natR h-*	This study
JM1598	*blt1-mCherry::natR h-*	
JM1603	*blt1(1-575)-mCherry::natR mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- + pSM26 (pmid GFP-Nter mid1 1-506, leu1+) integrated*	This study
JM1607	*blt1(1-575)-mEGFP::kanMX6 h-*	This study
JM1642	*blt1(1-575)-mEGFP cdr2Δ::ura4+ ura4-D18 h-*	This study
JM1652	*blt1Δ::kanMX6 cdr2-3HA::natR leu1-32*	This study
JM1680	*mid1Δ::natR blt1Δ::kanMX6 ura4-294 leu1-32 + pJK210 (ura4+) and pSM26(Mid1GFP-Nter, leu1+) integrated*	This study
JM1681	*mid1Δ::natR blt1Δ::kanMX6 ura4-294 leu1-32 + pJM584 (pblt1-Blt1-mCherry, ura4+) and pSM26(Mid1GFP-Nter, leu1+) integrated*	This study
JM1682	*mid1Δ::natR blt1Δ::kanMX6 ura4-294 leu1-32 + pJM585 (pblt1-blt1Δ1-mCherry, ura4+) and pSM26(Mid1GFP-Nter, leu1+) integrated*	This study
JM1684	*blt1Δ::natR ade6-M210 ura4-294 + pJM584 (pblt1-Blt1-mCherry, ura4+) integrated*	This study
JM1685	*blt1Δ::natR ade6-M210 ura4-294 + pJM585 (pblt1-blt1Δ1-mCherry, ura4+) integrated*	This study
JM2193	*blt1Δ::natR cdr2-mEGFP::kanMX6 ura4-294 + pJM584 (pblt1-Blt1-mCherry, ura4+) integrated*	This study
JM2194	*blt1Δ::natR cdr2-mEGFP::kanMX6 ura4-294 + pJM585 (pblt1-blt1Δ1-mCherry, ura4+) integrated*	This study
JM2195	*blt1Δ::natR gef2-mEGFP::kanMX6 ura4-294 + pJM584 (pblt1-Blt1-mCherry, ura4+) integrated*	This study
JM2196	*blt1Δ::natR gef2-mEGFP::kanMX6 ura4-294 + pJM585 (blt1Δ1-mCherry, ura4+) integrated*	This study
JM2197	*blt1Δ::natR cdr2Δ::kanMX6 ura4-294 + pJM584 (pblt1-Blt1-mCherry, ura4+) integrated*	This study
JM2198	*blt1Δ::natR cdr2Δ::kanMX6 ura4-294 + pJM585 (pblt1-blt1Δ1-mCherry, ura4+) integrated*	This study
AP528	*mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- +pAP146 (pmid1-mid1-GFP, leu1+) integrated*	(2)
AP583	*mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- +pAP159 (pmid1-helix* mid1-GFP,leu1+) integrated*	(2)
AP998	*mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- + pSM26 (pmid GFP-Nter mid1 1-506, leu1+) integrated*	(2)
AP1889	*mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- + pMA15 (pmid1-GFP-mid1-300-350, leu1+) integrated*	This study
AP2147	*blt1Δ::kanMX6 mid1 Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h+ + pMA15 (pmid1-GFP-mid1-300-350, leu1+) integrated*	This study

AP2165	*blt1-mCherry::natR mid1Δ::kanMX4 ade6-M216 leu1-32 ura4-D18 +pSM26 (pmid GFP-Nter mid1 1-506, leu1+) integrated*	This study
AP2172	*blt1Δ::kanMX6 mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h+ +pSM26 (pmid GFP-Nter mid1 1-506, leu1+) integrated*	This study
AP2335	*blt1Δ::kanMX6 mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h+ + pAP159 (pmid1-helix* mid1-GFP,leu1+) integrated*	This study
AP3177	*cdr2-GFP::kanMX6 ade6-M210 ura4-D18 leu1-32 h-*	This study
AP3490	*blt1-mEGFP::kanMX6 mid1Δ::ura4+ ade6-M210 leu1-32 ura4-D18 h- + pAP93 (pmid1-mid1, leu1+) integrated*	This study
AP3491	*blt1-mEGFP::kanMX6 mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- + pMA34 (pmid1-mid1Δ300-350) integrated*	This study
AP3645	*blt1-mEGFP::kanMX6 mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- pMG60 (pmid1-GST-mid1-300-450, leu1+) integrated*	This study
AP3866	*blt1-mEGFP::kanMX6 gef2Δ::natR mid1Δ::ura4+ ade6-M21X leu1-32 ura4-D18 h- pMG60 (pmid1-GST-mid1-300-450, leu1+) integrated*	This study
AP3867	*gef2-mEGFP::kanMX6 blt1Δ::natR mid1Δ::ura4+ ade6-M21X leu1-32 ura4-D18 h+ pMG60 (pmid1-GST-mid1-300-450, leu1+) integrated*	This study
AP3868	*gef2-mEGFP::kanMX6 mid1Δ::ura4+ ade6-M21X leu1-32 ura4-D18 h- pMG60 (pmid1-GST-mid1-300-450, leu1+) integrated*	This study
AP3872	*gef2Δ::natR blt1-mEGFP::kanMX6 ade6-M210 leu1-32 ura4-D18 h-*	This study
AP3873	*blt1Δ::natR gef2-mEGFP::kanMX6 ade6-M216 leu1-32 ura4-D18 h+*	This study
AP3898	*cdr2-GFP-CAAX::kanMX6 ade6-M216 leu1-32 ura4-D18 h+*	This study
AP3900	*rlc1-mcherry::natR mid1Δ::ura4+ ade6-M216 ura4-D18 leu1-32 h- +pSM26 (pmid GFP-Nter mid1 1-506, leu1+) integrated*	This study
AP3901	*rlc1-mcherry::natR mid1Δ::ura4+ ade6-M216 ura4-D18 leu1-32 h- +pMA15 (pmid1-GFP-mid1-300-350, leu1+) integrated*	This study
AP3902	*rlc1-mcherry::natR mid1Δ::ura4+ ade6-M216 ura4-D18 leu1-32 h- +pMA10 (pmid1-GFP-Ntermid1Δ300-350, leu1+) integrated*	This study
AP3903	*blt1Δ::kanMX6 rlc1-mcherry::natR mid1Δ::ura4+ ade6-M216 ura4-D18 leu1-32 h+ +pSM26 (pmid GFP-Nter mid1 1-506, leu1+) integrated*	This study
AP3906	*blt1-mEGFP::kanMX6 rlc1-mCherry::natR sfi1-mRFP::kanMX6 leu1-32 ura4-D18 h-*	This study
AP3907	*blt1(1-575)-mEGFP::kanMX6 rlc1-mCherry::natR sfi1-mRFP::kanMX6 ura4-D18 h-*	This study
AP3909	*cdr2-GFP-CAAX::kanMX6 blt1(1-575)-mCherry::natR mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h- +pSM26 (pmid GFP-Nter mid1 1-506, leu1+) integrated*	This study
AP3910	*rlc1-mCherry::natR blt1Δ::kanMX6 mid1Δ::ura4+ ura4-D18 leu1-32 ade6-M216 + pMA15 (pmid1-GFP-mid1-300-350, leu1+) integrated h+*	This study
AP3924	*mid1-Nter(1-506)::hphMX blt1-mEGFP::kanMX6 rlc1-mCherry::natR sfi1-mRFP::kanMX6 leu1-32 ura4-D18 h-*	This study
AP3925	*mid1-Nter(1-506)::hphMX blt1(1-575)-mEGFP::kanMX6 rlc1-mCherry::natR sfi1-mRFP::kanMX6 ura4-D18 h-*	This study

References for strain table:

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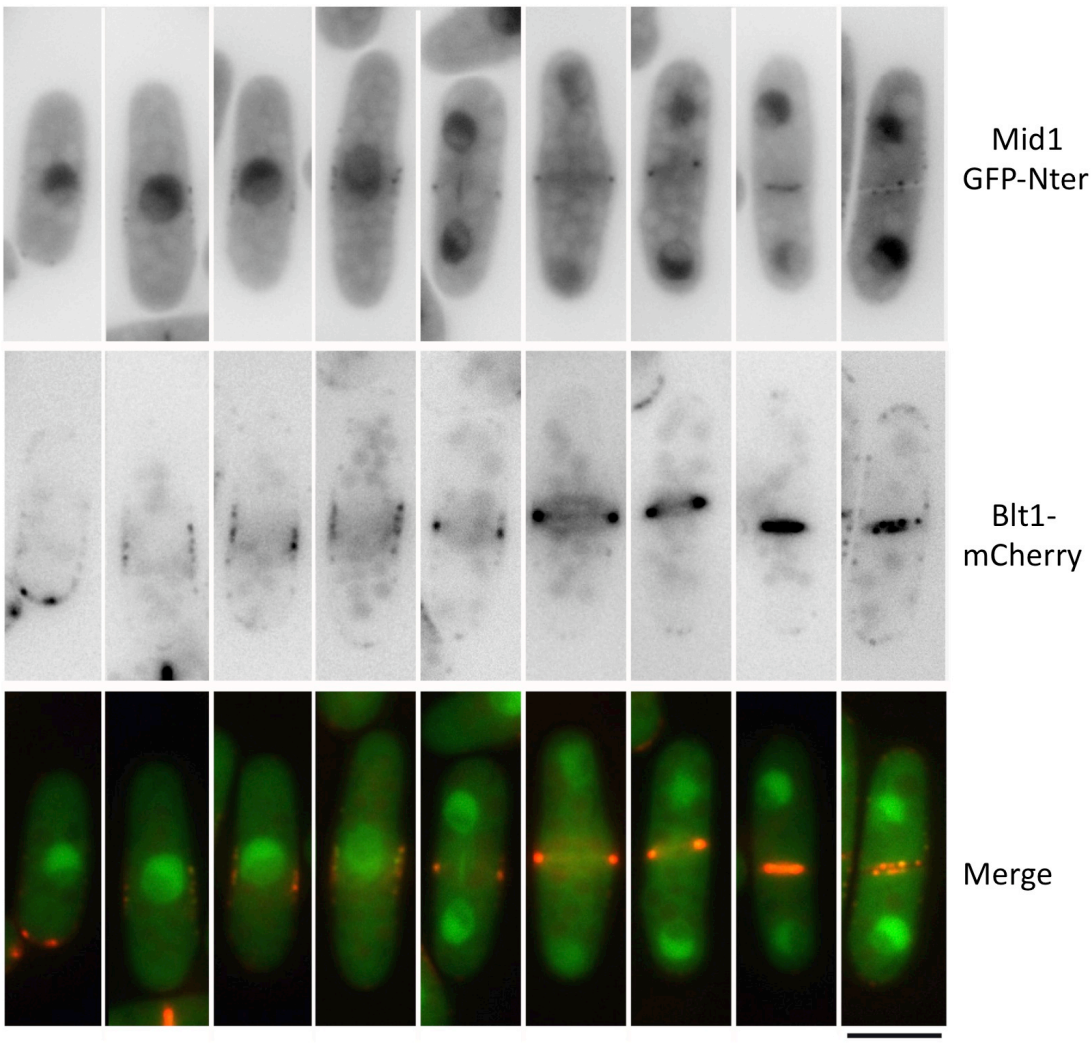


Figure S1

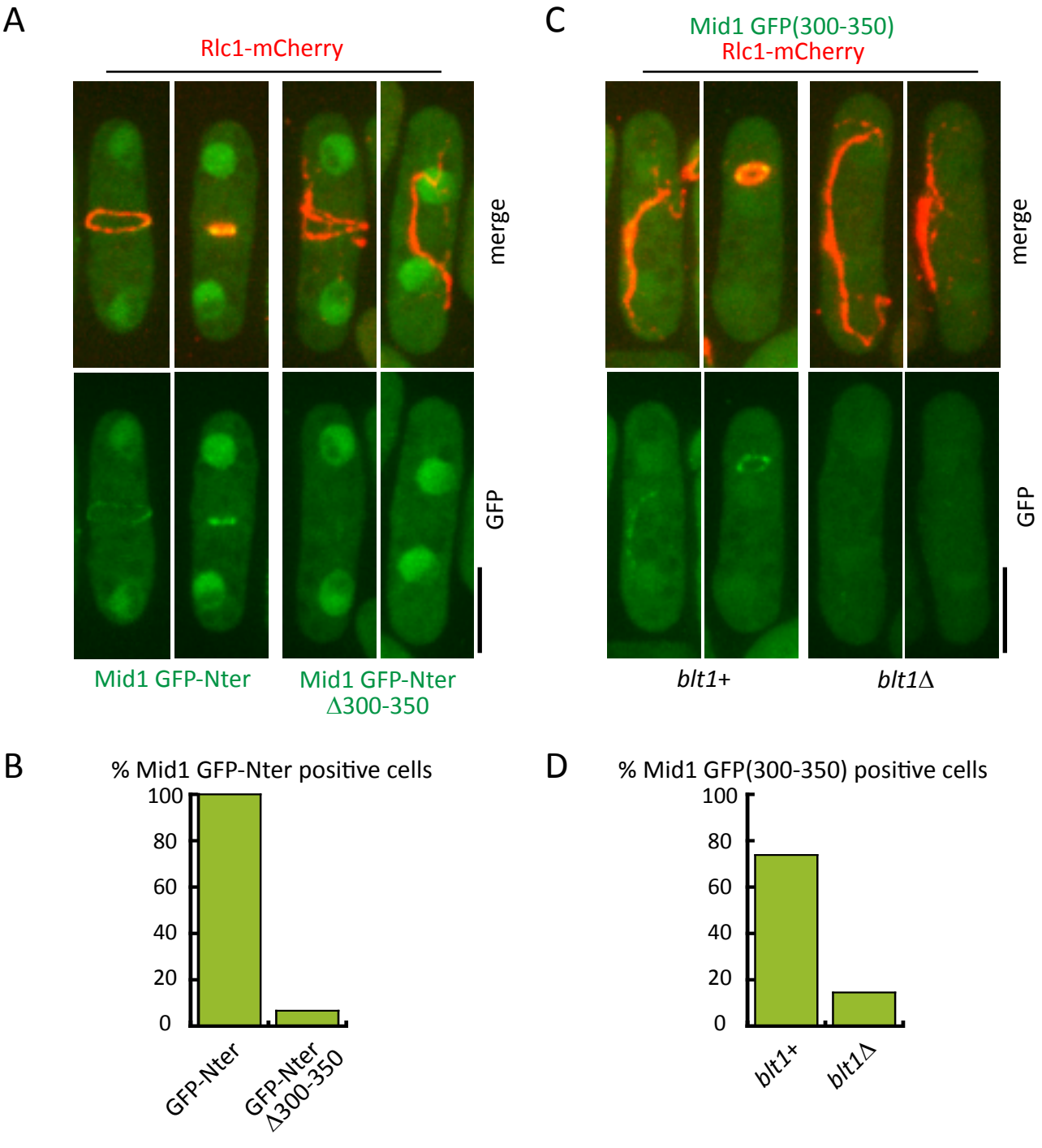

A
Rlc1-mCherry
merge
GFP
Mid1 GFP-Nter
Mid1 GFP-Nter
Δ300-350
C
Mid1 GFP(300-350)
Rlc1-mCherry
merge
GFP
blt1+
blt1Δ
B
% Mid1 GFP-Nter positive cells
100
80
60
40
20
0
GFP-Nter
GFP-Nter
Δ300-350
D
% Mid1 GFP(300-350) positive cells
100
80
60
40
20
0
blt1+
blt1Δ

Figure S2

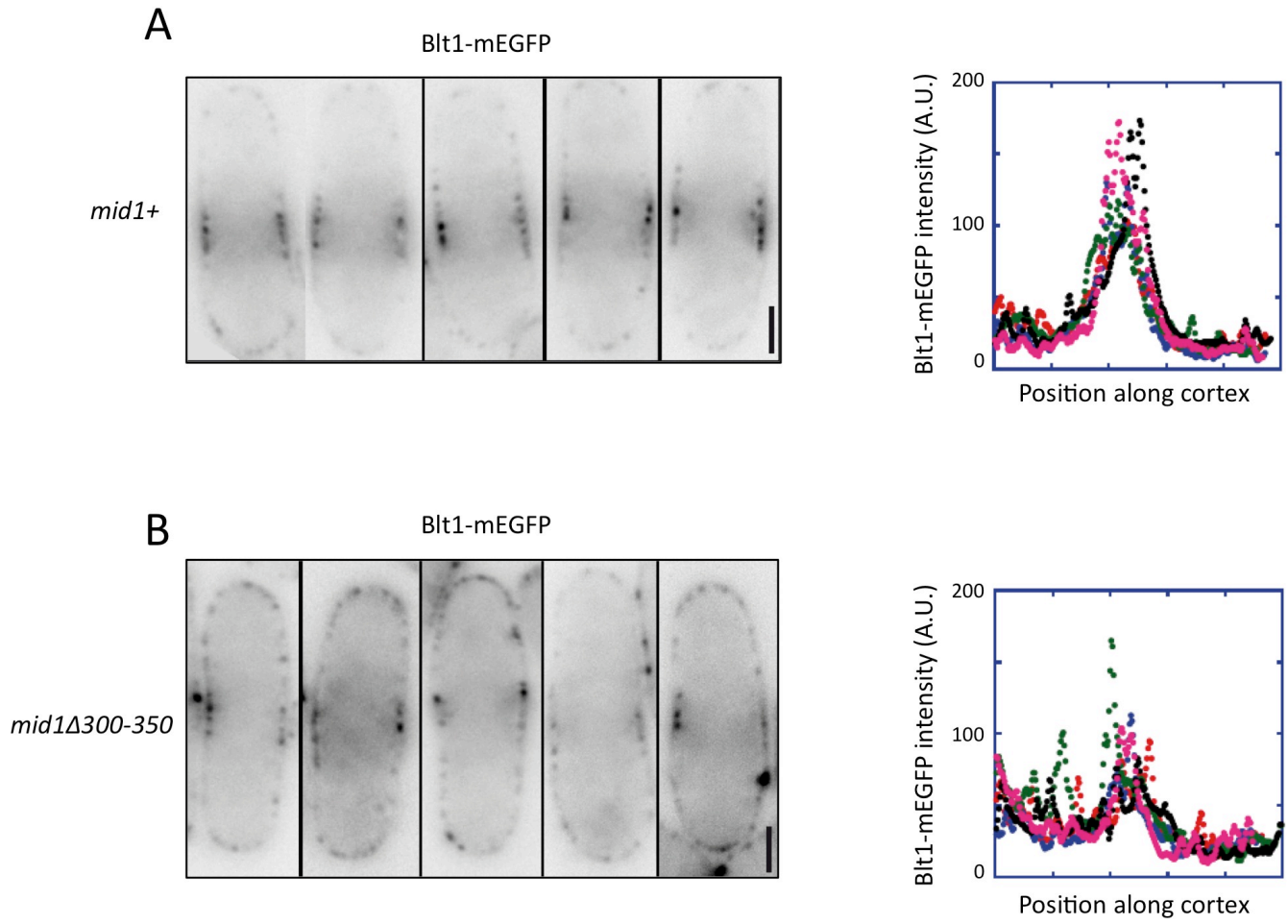

A
Blt1-mEGFP
mid1+
Blt1-mEGFP intensity (A.U.)
200
100
0
Position along cortex
B
Blt1-mEGFP
mid1Δ300-350
Blt1-mEGFP intensity (A.U.)
200
100
0
Position along cortex

Figure S3

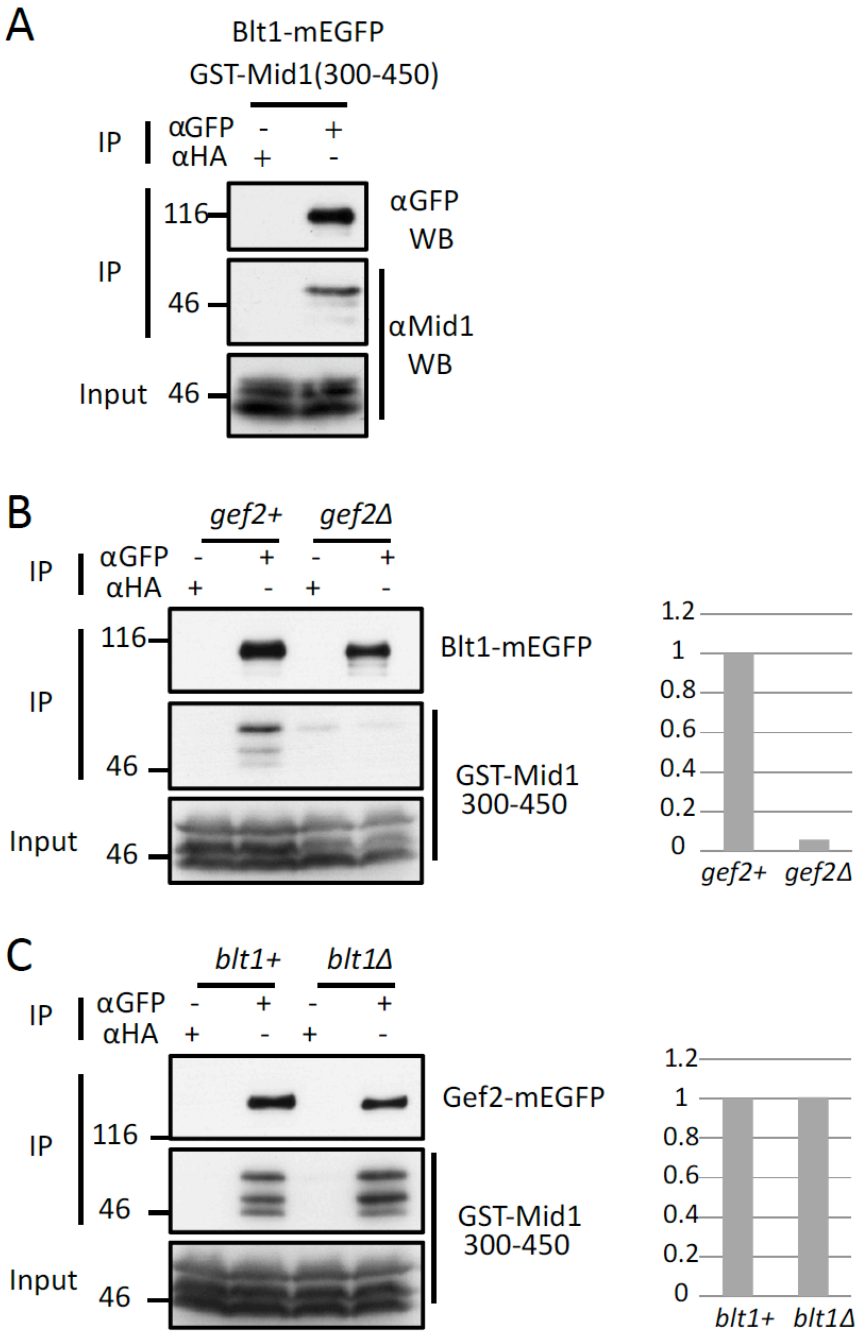

A
Blt1-mEGFP
GST-Mid1(300-450)
IP
αGFP - +
αHA + -
116
αGFP
WB
IP
46
αMid1
WB
Input 46
B
gef2+ gef2Δ
IP
αGFP - + - +
αHA + - + -
116
Blt1-mEGFP
IP
46
GST-Mid1
300-450
Input 46
1.2
1
0.8
0.6
0.4
0.2
0
gef2+ gef2Δ
C
blt1+ blt1Δ
IP
αGFP - + - +
αHA + - + -
Gef2-mEGFP
IP
116
46
GST-Mid1
300-450
Input 46
1.2
1
0.8
0.6
0.4
0.2
0
blt1+ blt1Δ

Figure S4

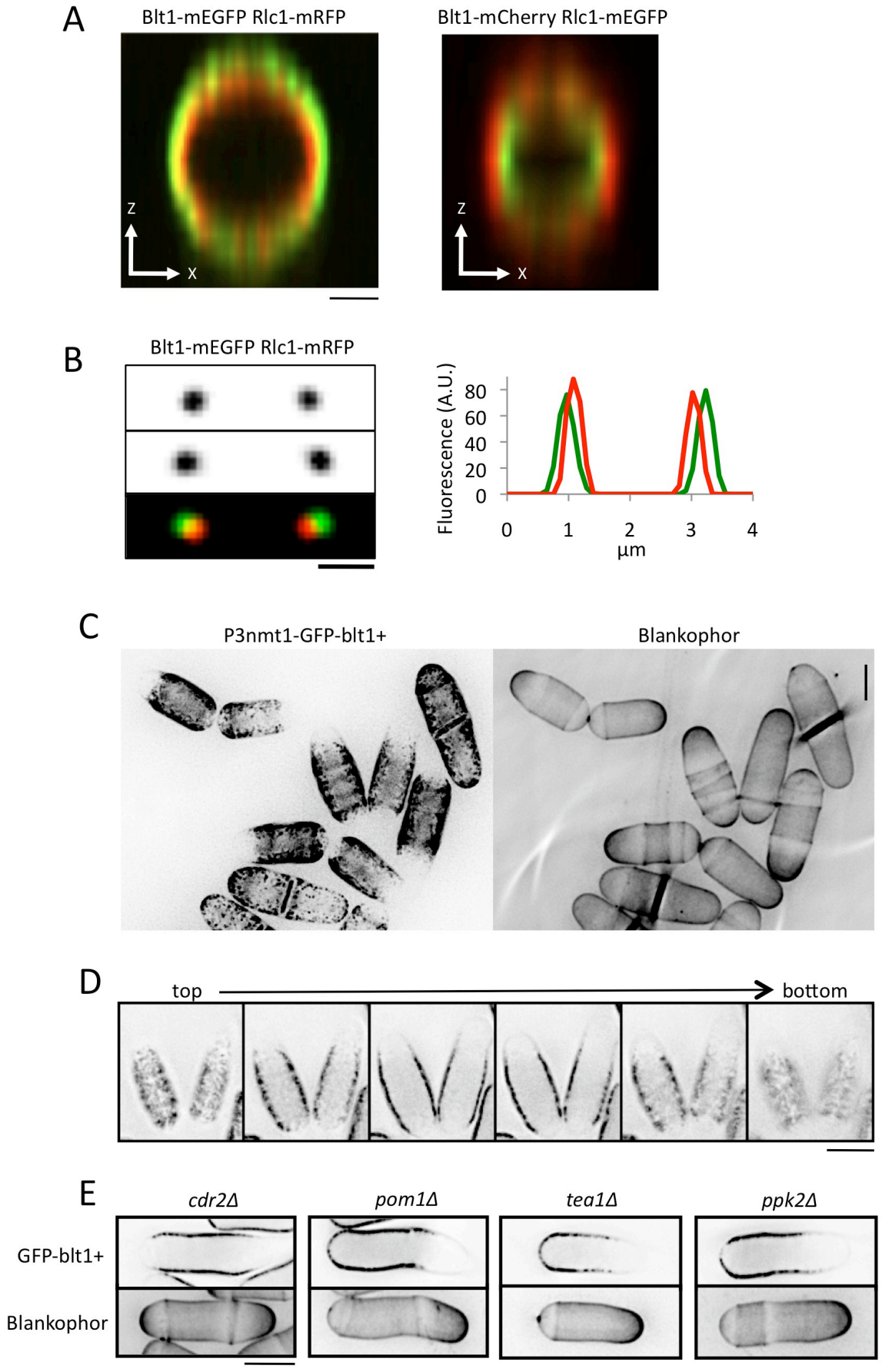
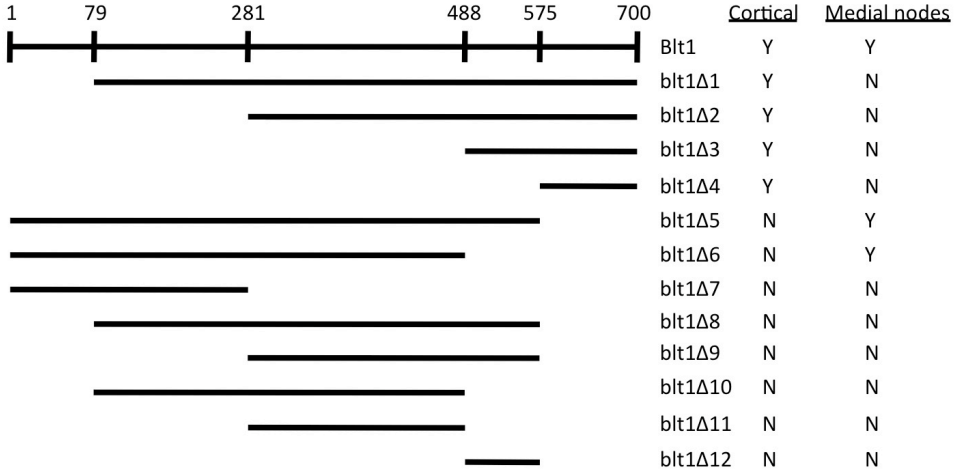

A
Blt1-mEGFP Rlc1-mRFP
Blt1-mCherry Rlc1-mEGFP
z
x
B
Blt1-mEGFP Rlc1-mRFP
Fluorescence (A.U.)
80
60
40
20
0
0
1
2
3
4
µm
C
P3nmt1-GFP-blt1+
Blankophor
D
top
bottom
E
cdr2Δ
pom1Δ
tea1Δ
ppk2Δ
GFP-blt1+
Blankophor

Figure S5

A


1
79
281
488
575
700
Cortical
Medial nodes
Blt1 Y Y
blt1Δ1 Y N
blt1Δ2 Y N
blt1Δ3 Y N
blt1Δ4 Y N
blt1Δ5 N Y
blt1Δ6 N Y
blt1Δ7 N N
blt1Δ8 N N
blt1Δ9 N N
blt1Δ10 N N
blt1Δ11 N N
blt1Δ12 N N

B

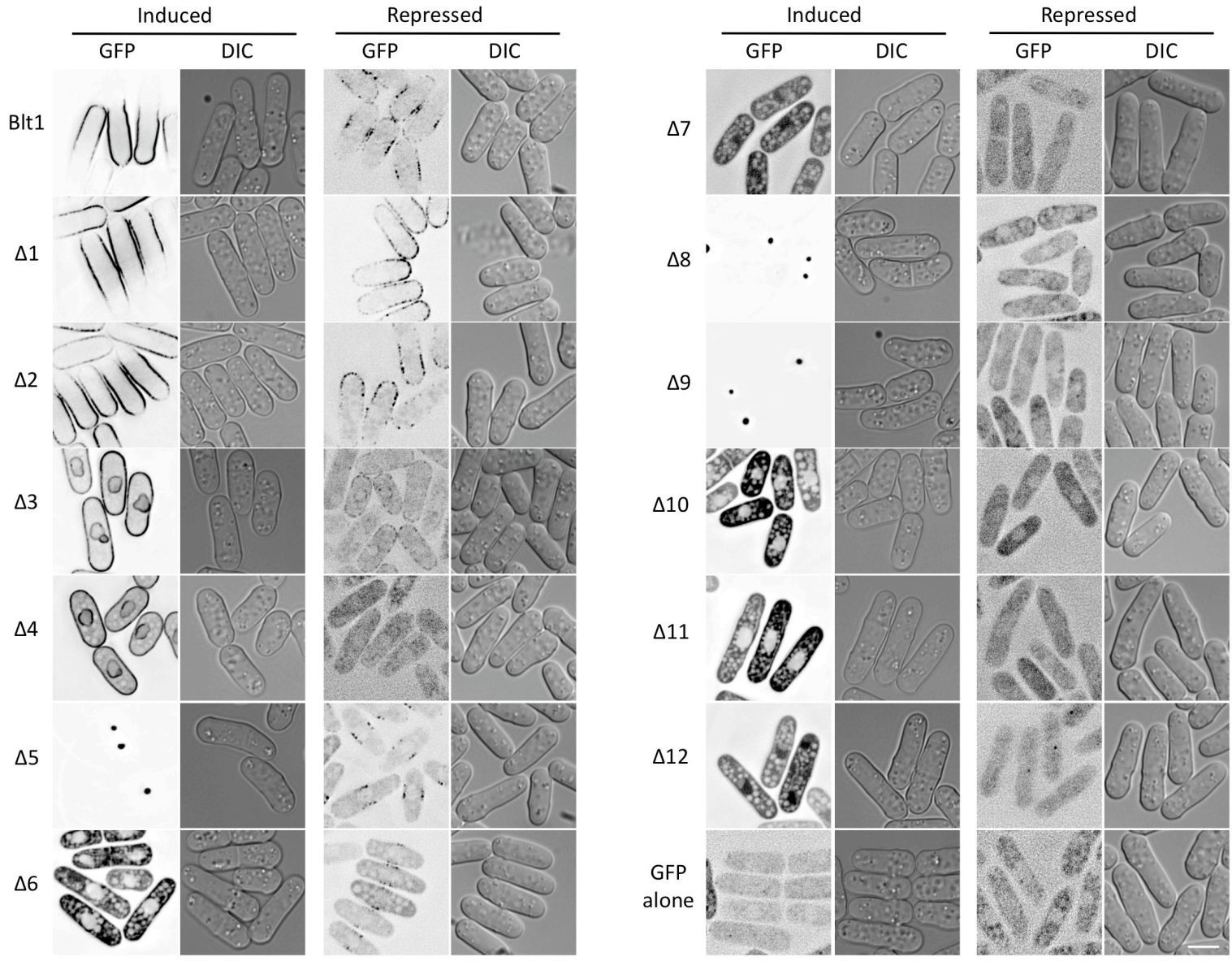

Induced
Repressed
GFP
DIC
Blt1
Δ1
Δ2
Δ3
Δ4
Δ5
Δ6
Δ7
Δ8
Δ9
Δ10
Δ11
Δ12
GFP alone

Figure S6

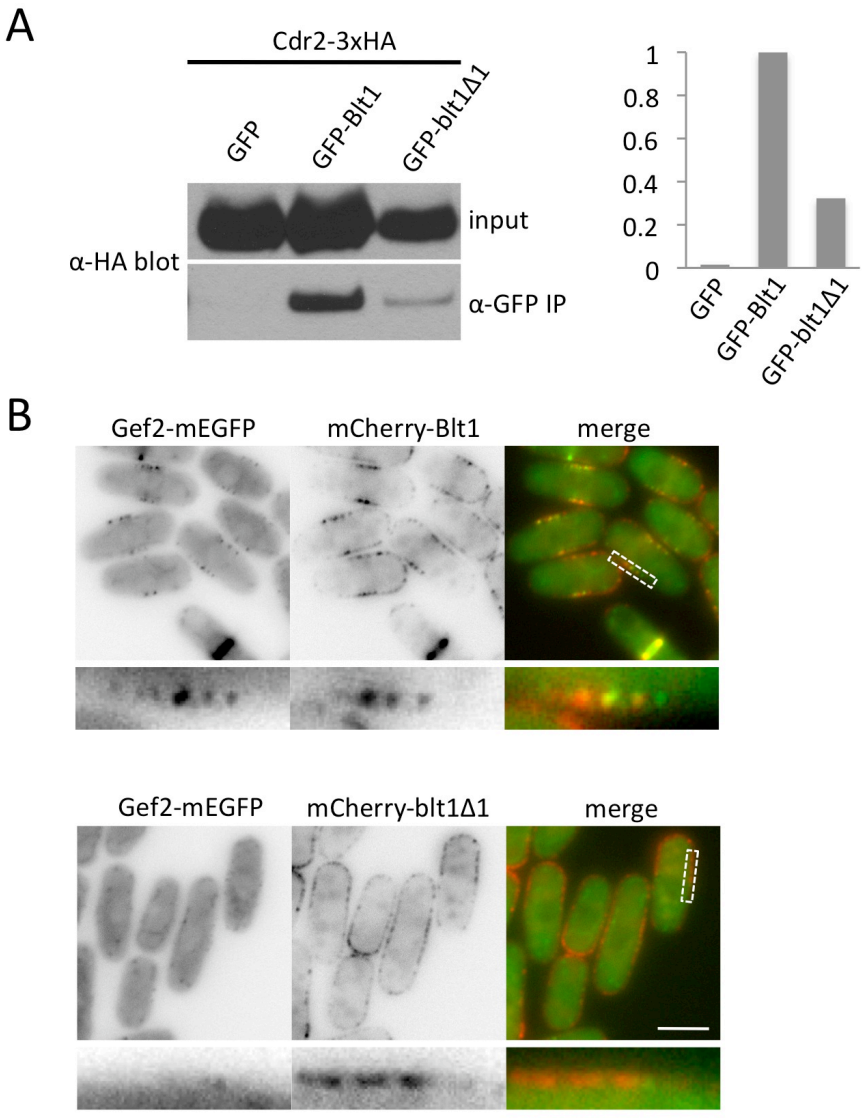

A
Cdr2-3xHA
GFP
GFP-Blt1
GFP-blt1Δ1
α-HA blot
input
α-GFP IP
1
0.8
0.6
0.4
0.2
0
GFP
GFP-Blt1
GFP-blt1Δ1
B
Gef2-mEGFP
mCherry-Blt1
merge
Gef2-mEGFP
mCherry-blt1Δ1
merge
C
gef2-mEGFP mCherry-blt1Δ1
Gef2-mEGFP
DIC

Figure S7

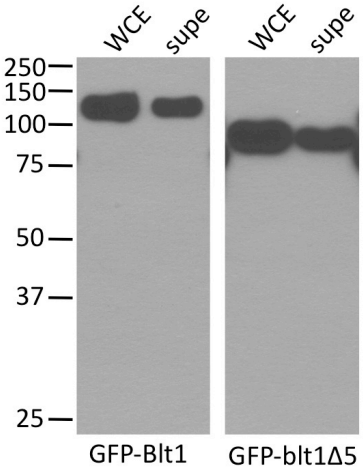
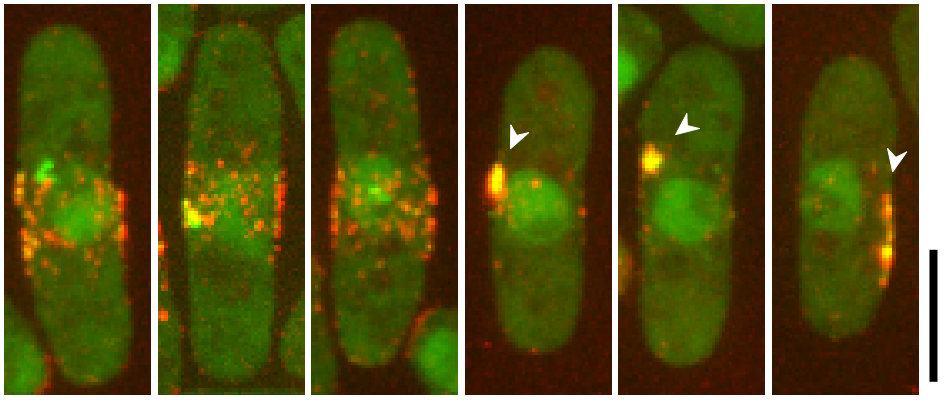


Figure S8



GFP-Mid1-Nter
Blt1-mCherry

GFP-Mid1-Nter
blt1-Δ5-mCherry

FigureS9

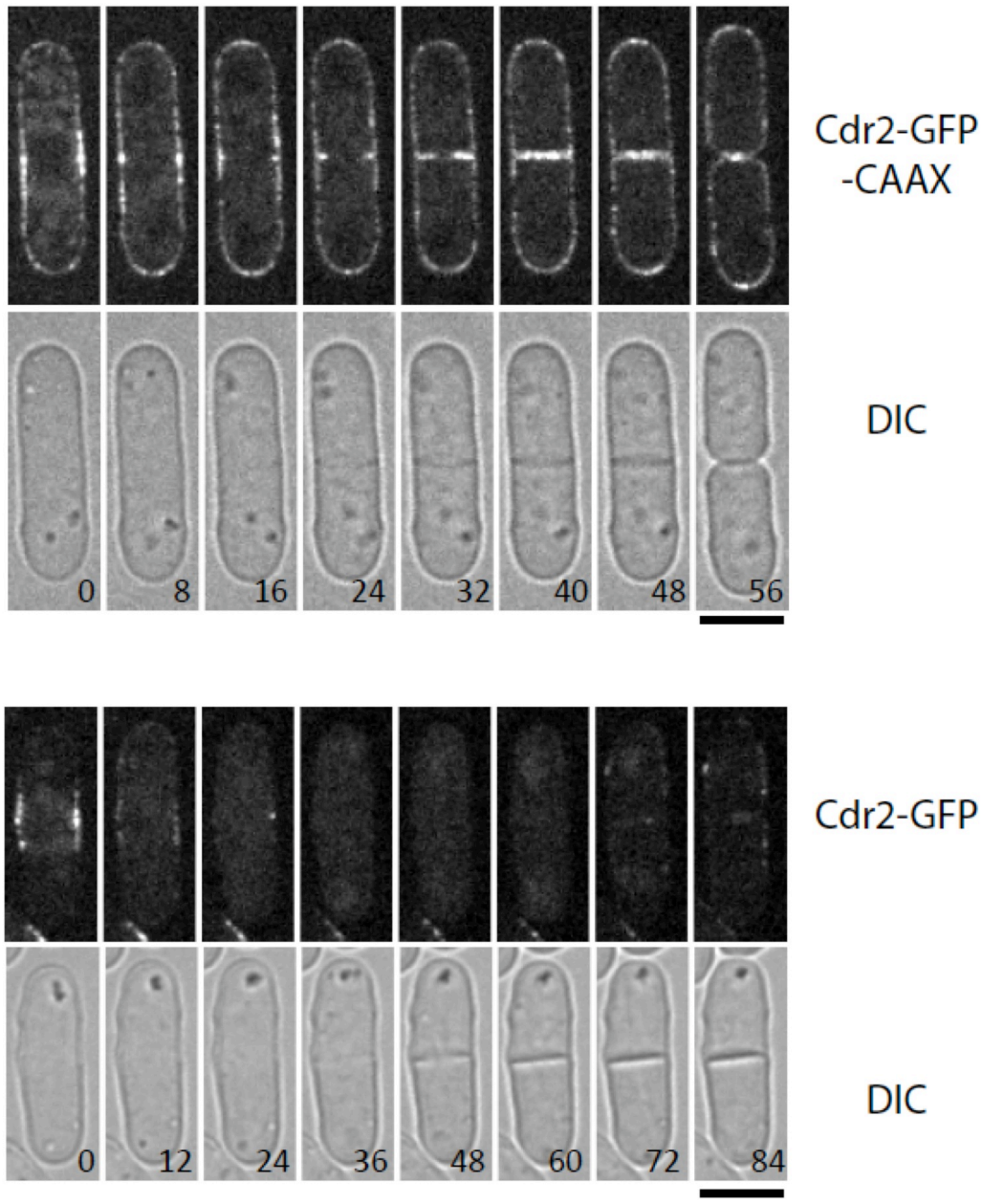

Cdr2-GFP
-CAAX
DIC
0
8
16
24
32
40
48
56
Cdr2-GFP
DIC
0
12
24
36
48
60
72
84

Figure S10

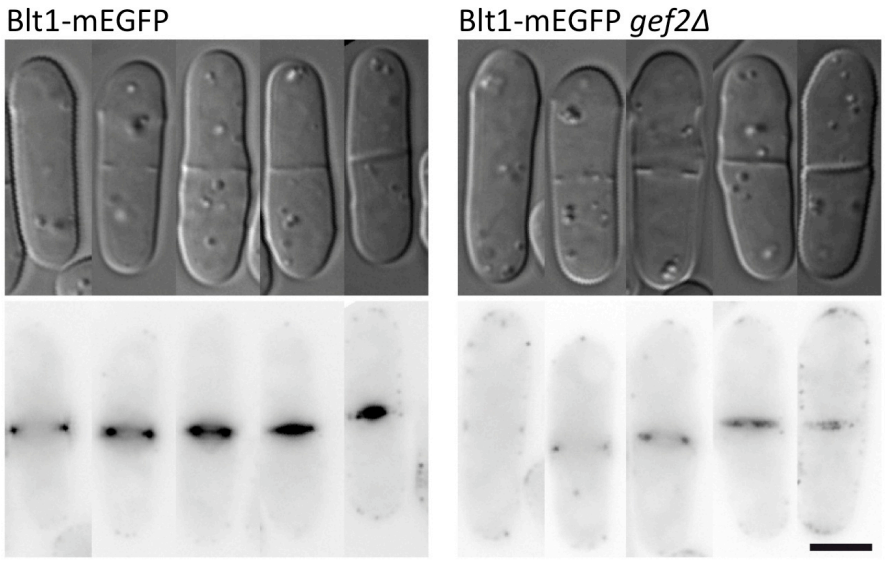


Figure S11

B. Cdr2 node organization and architecture.

1. Background

As explained earlier in this manuscript, the existence of a cell size homeostasis system was proposed many years ago in budding and fission yeast. In 2009, a first molecular model linking mitosis entry to cell size was proposed in parallel by the groups of Paul Nurse and Sophie Martin (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009). It involved the CGN, a pathway controlled by two antagonistic kinases. The DYRK kinase Pom1 forming gradients diffusing from the cell tips, that phosphorylates and inhibits the SAD kinase Cdr2, forming a large band of medial cortical nodes. These nodes were shown to be active sites for Wee1 inhibition, influencing the timing of mitotic entry. Cdr2 node activity towards Wee1 involves Cdr1, the second SAD kinase of fission yeast associated with Cdr2 within medial nodes. These two kinases were known to phosphorylate Wee1 and Cdr1 was shown to inhibit Wee1 activity in vitro (Russell and Nurse 1987; Young and Fantes 1987; Coleman, Tang et al. 1993; Parker, Walter et al. 1993; Wu and Russell 1993; Breeding, Hudson et al. 1998; Kanoh and Russell 1998). It was proposed that in short cells the concentration of Pom1 at the cell middle is high enough to inhibits Cdr2, delaying mitotic entry, but as the cell grow longer, Pom1 concentration at the cell middle diminishes, allowing Cdr2 activation, Wee1 inhibition and mitotic entry. The CGN was also shown to control the assembly of precursor nodes for the contractile ring through Mid1 and promote thereby medial division (Almonacid, Moseley et al. 2009).

Several questions remained open after these publications. In particular, how Pom1 controls Cdr2 activity was unknown and whether Pom1 inhibited only Cdr2 localization to nodes with impact on its activity or affected both its localization and activity independently remained an open question.

Recent publications provided complete or partial answers to these questions as described in the introduction. Briefly, we now know how Pom1 regulates the assembly of Cdr2 nodes as well as its activity (Bhatia, Hachet et al. 2014; Rincon, Bhatia et al. 2014). Pom1 reduces Cdr2 affinity for lipids as well as clustering properties to disfavor Cdr2 nodes assembly in the cell tips where Pom1 is highly concentrates. Pom1 blocks the activation of Cdr2 kinase activity by inhibiting the phosphorylation of the T-loop by the CaMKK kinase Ssp1 (Deng, Baldissard et al. 2014).

Another unanswered question was how Cdr2 was controlling the recruitment of various node components involved in Wee1 regulatory pathway or cytokinesis. And how Cdr2 did control Cdr1-dependent inactivation of Wee1? During the second part of my thesis, I have studied Cdr2 nodes architecture in order to understand how the signaling pathway for Wee1 inhibition functions. I have found that Wee1 and Mid1, the two main effectors of Cdr2 for cell cycle and cytokinetic related functions are recruited by Cdr2 UBA domain in a kinase activity dependent manner while Cdr1 associates with Cdr2 C-terminal domain. Mid1 also binds to Cdr2-Cter domain and may bridge the N- and C-terminal domains together, while Blt1 associates with Cdr2 internal spacer. Our results suggest that the association of Cdr2 effectors with different Cdr2 domains may constrain Cdr1 and Wee1 spatially to promote Wee1 inhibition upon activation of Cdr2 kinase.

Article 2: Molecular control of the Wee1 regulatory pathway by the SAD kinase Cdr2

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IV.
Discussion

IV. Discussion and perspectives

Choosing the correct division plane for division and the good moment to enter mitosis are two important factors to ensure cell size homeostasis. The existence of cell size homeostasis mechanisms was first described in yeast in the 70s. Fission yeast has remained since a very good model to study cell cycle progression and coordination with growth.

The work of this thesis has focused on the study of fission yeast medial cortical nodes that play a role in both division plane positioning and mitotic commitment.

The assembly of medial cortical nodes

The medial cortical nodes are oligomeric protein complexes organized by the SAD kinase Cdr2 (Moseley, Mayeux et al. 2009). A recent study from out laboratory has revealed that Cdr2 possesses two essential properties to self-organize into nodes: 1/ membrane binding that happens through its C-terminal KA1 domain and a stretch of basic aminoacids adjacent to it. Both elements establish electrostatic interactions with acidic phospholipids in plasma membrane. 2/ clustering properties through its KA-1 domain. Clustering is reinforced by the N-terminal region of the protein with the help of Mid1. Cdr2 clustering and interaction with Mid1 are negatively regulated by Pom1 to restrict the nodes to the medial cortex(Rincon, Bhatia et al. 2014).

The nodes composition is complex. So far 8 components have been identified Cdr2, Mid1, Wee1, Cdr1, Blt1, Gef2, Klp8 and Nod1. Among those, two, Blt1 and Mid1, have their own membrane binding domain and exhibit oligomerization properties similar to Cdr2. Mid1 possesses a lipid-binding amphipathic helix (Celton-Morizur, Bordes et al. 2004) and may assemble in octamers based of biochemical studies on a fragment of Mid1 (Saha and Pollard 2012). Our work on Blt1 showed that it contains a C-terminal basic region involved in lipid

binding (Guzman-Vendrell, Baldissard et al. 2013) and Blt1 was recently shown to assemble into tetramers (Goss, Kim et al. 2014).

These properties allow Mid1 and Blt1 to assemble into node-like structures on the cortex independently of Cdr2. Accordingly, Mid1 can form cytokinetic nodes, ring percursors, at mitotic entry, after export from the nucleus, in the absence of Cdr2. These nodes are fully competent to recruit the components of the contractile ring and induce contractile ring. Nuclear export at mitotic entry ensures their regionalization in the cell middle and permits to efficiently place the contractile ring in the cell middle (Almonacid, Moseley et al. 2009).

Blt1 also forms node-like structures independently of Cdr2 (Moseley, Mayeux et al. 2009). These nodes, called recently type II nodes by the laboratory of Tom Pollard, as compared to Cdr2/Mid1 type I nodes, are observed close to the cell tip in very short G2 cells in presence of Cdr2 or throughout interphase in its absence (Moseley, Mayeux et al. 2009). They recruit Gef2 and Nod1, that share sequence similarity and interact with one another through their homologous C-terminal region (Jourdain, Brzezinska et al. 2013; Zhu, Ye et al. 2013), as well as Klp8 (Akamatsu, Berro et al. 2014). Like Blt1, Gef2, Nod1 and Klp8 compact with medial cortical nodes into the contractile ring and remain associated with it during ring constriction.

A recent study has suggested that in wild type cells, medial cortical nodes are actually formed by fusion of these two kinds of nodes. Blt1 nodes seem to be released from the contractile ring as it disassembles at the end of cytokinesis and were proposed to diffuse from the new end of the cell towards the cell middle where Cdr2 nodes containing Mid1 reassemble. The two node types were postulated to fuse to form the medial cortical nodes (Akamatsu, Berro et al. 2014). Nevertheless, long movements of Blt1 nodes were not observed and this hypothesis cannot account for the fact that Blt1 nodes remain at the cell tips in the absence of Cdr2 (Moseley, Mayeux et al. 2009). Therefore, we favor an alternative model in which Blt1 nodes from the

previous division disintegrate as Bl1 is incorporated together with Nod1, Gef2 and Klp8 in new medial cortical nodes, in a competition driven process.

During the development of this thesis work, we realized that interactions between the different node components are really complex. Because Cdr2 is the key organizer of the medial cortical nodes, our simple hypothesis when we began this work was that Cdr2 may scaffold node components independently from one another. It is clearly not the case since lots of dependencies have been discovered between components for their correct association with medial cortical nodes.

Blt1 depends on Cdr2 for node localization but Blt1 localization to nodes is partially dependent on Mid1 (Moseley, Mayeux et al. 2009). We have shown that Blt1 is in turn important to stabilize Mid1 association with Cdr2 when it is deficient for lipid-binding (Guzman-Vendrell, Baldissard et al. 2013). Gef2 and Nod1 have interdependency in localization (Jourdain, Brzezinska et al. 2013; Zhu, Ye et al. 2013), and Gef2 seems to be mediating Blt1 interaction with Mid1 (Ye, Lee et al. 2012; Guzman-Vendrell, Baldissard et al. 2013). During interphase Gef2 and Nod1 localization depends on Blt1 but during cytokinesis, when Cdr2 becomes cytoplasmic (Morrell, Nichols et al. 2004; Moseley, Mayeux et al. 2009; Akamatsu, Berro et al. 2014), Blt1 association with the contractile ring now relies on the Gef2/Nod1 complex that interacts with the F-Bar protein Cdc15 (Guzman-Vendrell, Baldissard et al. 2013; Jourdain, Brzezinska et al. 2013; Zhu, Ye et al. 2013).

One puzzling discovery made during this thesis work is the complexity of the interactions between Cdr2 and Mid1. We can count up to three interaction points between Mid1 and Cdr2. The first and major interaction is between Cdr2 UBA and Mid1 400-450 region ((Almonacid, Moseley et al. 2009), Results from Article 2). The second involves Blt1 that associates with Cdr2 spacer region and mediates interactions with Mid1 300-350 region through Gef2 ((Ye, Lee et al. 2012; Guzman-Vendrell, Baldissard et al. 2013); Results from Article 2). The third

interaction involves the C-terminal region of Cdr2. Whether this interaction is direct or not is not known and the region of Mid1 involved has not been tested yet. Since Cdr2 C-terminus associated with the plasma membrane, we hypothesize that this interaction may involve Mid1 PH domain that was shown to reinforce Mid1 membrane binding (Lee, Coffman et al. 2012). Why do Cdr2 and Mid1 require such a tight and redundant interaction mode is still a mystery to us.

In conclusion, the complexity and large interdependency of interactions between its components suggests that medial nodes should form a tight protein complex rather than as a simple scaffold-based signaling platform.

Cytokinetic functions of medial cortical nodes

The primary cytokinetic function of medial cortical nodes is to serve as precursors for the contractile ring. Although cells can cope with the absence of these precursors (Huang, Yan et al. 2008), their presence confers efficiency and robustness to the process of contractile ring assembly that might be key for fission yeast fitness under competition.

The robustness of contractile ring assembly is ensured by a high level of redundancy in the molecular mechanism involved. A good example of this redundancy comes from the analysis of the function of membrane anchors from different node component. Affecting the membrane-binding domain of Cdr2, Mid1 or Blt1 does not have major effects on cytokinesis. On the contrary, if we combine a Mid1 membrane-binding mutant with a Blt1 mutants defective for membrane-binding, cells have severe cytokinesis defects (Guzman-Vendrell, Baldissard et al. 2013). These studies reveal that a minimum of two membrane anchors is necessary to stabilize nodes at the medial cortex to promote contractile ring assembly but a third membrane anchor exists nevertheless.

A simple explanation for the need of several membrane anchors is that membrane binding is performed by domains with low affinity for lipids requiring homo or hetero-oligomerization to confer a higher avidity for membrane lipids.

Another explanation is the differential behavior of node components during cell cycle progression: Cdr2 that initiates nodes assembly leaves the nodes at the beginning of mitosis; Mid1 that recruits contractile ring components quits the ring before it constricts. Blt1 that joins the nodes during G2 stays associated with the ring as it constricts and may ensure the continuity of membrane anchoring throughout cytokinesis during which the F-Bar protein Cdc15 also contributes to membrane binding.

Interestingly, imaging revealed that Blt1, Gef2 and Nod1 form a ring slightly external to the acto-myosin ring (Guzman-Vendrell, Baldissard et al. 2013; Jourdain, Brzezinska et al. 2013), fitting their role as a membrane-anchoring complex for the contractile ring.

Another interesting fact is the presence of a GEF in the nodes and the ring. Gef2 may be a Rho GEF since it was shown to interact with in vitro with Rho1, Rho 4 and Rho5 (Zhu, Ye et al. 2013).

In animal cells RhoA has a key role for the assembly and contraction of the cytokinetic ring by activation of the formin mDia and of the Rho kinase ROCK that will in turn activate myosin II (see reviews (Fededa and Gerlich 2012; Green, Paluch et al. 2012)). Furthermore, the RhoA GEF ECT2 as well as RhoA has been shown to interact with anillin in humans that also scaffolds F-actin and Myosin II. And this interaction was shown to stabilize central spindle microtubules at the cortex during cytokinesis. This may influence the position of the contractile ring by stabilizing microtubule-cortical interactions at the division plane to ensure the generation of active RhoA in a discrete zone (Piekny and Glotzer 2008).

In fission yeast, the major protein controlling contractile ring assembly is the polo kinase Plo1. Ye and collaborators showed that Gef2 may function in parallel to Plo1 to promote ring assembly. Thus, interactions between anillin-like proteins, RhoA and its regulators may be evolutionary conserved although their functional role during cytokinesis may be minored in presence of strong Plo1-dependent pathways for ring assembly (Ye, Lee et al. 2012).

The mitotic promoting function of medial cortical nodes

The second function of medial cortical nodes is to promote mitotic entry by inhibition of Wee1. In this thesis we have shown that Cdr2 binds Wee1 and Mid1 through its UBA domain and seen that their binding to Cdr2 UBA requires Cdr2 kinase activity. Reciprocally, we have found that Cdr2 UBA is necessary for Cdr2 activity.

Two hypotheses can account for these results. Either a change in conformation of Cdr2 UBA accompanies the expected change in conformation, from an open to a closed conformation, of the kinase domain upon activation of Cdr2. This may create binding sites for Wee1 and Mid1 on Cdr2 UBA. This hypothesis is supported by the reported interactions between the helix C of the N-lobe of the kinase domain, known to move during kinase activation, with the UBA/AID domains of AMPKs. The fact that these interactions are conserved in our model of Cdr2 KD+UBA which make this hypothesis a plausible one. Crystal structure of Cdr2 KD+UBA in an active and inactive form would address this point.

Alternatively, active Cdr2 may phosphorylate its UBA or the sites of interaction of Cdr2 on Wee1 and Mid1 to promote their binding. We can of course imagine that phosphorylations on both sides are required for binding. Due to steric problems, Cdr2 is unlikely phosphorylates its UBA intramolecularly, but Cdr2 oligomerization may allow phosphorylation of Cdr2 UBA by Cdr2 kinase domain in trans. However, the fact that Cdr2 was reported to phosphorylate a

Wee1 fragment in vitro (Wu and Russell 1993) rather favors the hypothesis that the phospho-regulatory events may take place on Wee1 side. In any case, mapping Cdr2-dependent phosphosites on Wee1, Mid1 and Cdr2 itself is required to solve this issue.

Cdr1, which has a strong similarity with Cdr2, also belongs to the AMPK family of kinases, but it is a peculiar AMPK in two ways. First, it does not have a KA1 domain, and relies for this reason on Cdr2 for a proper cortical localization. We have shown that Cdr1 association with Cdr2 actually depends on Cdr2 C-terminal domain also involved in membrane targeting and clustering. Second Cdr1 T-loop lacks the conserved threonine that needs to be phosphorylated for the activation of AMPKs. Since Cdr1 kinase activity and ability to inhibit Wee1 has been demonstrated (Coleman, Tang et al. 1993; Parker, Walter et al. 1993; Wu and Russell 1993), Cdr1 may be constitutively active unless bound to its inhibitors Skb1 and Nif1 through its UBA domain.

Strikingly, Blt1, Nod1 and Gef2 which localization and function discussed above indicate a role during cytokinesis, also have some impact on medial cortical nodes mitotic function since cells deficient for these factors all have longer cell size a division than wild type cells. How these node components modulate the Wee1 regulatory pathway remains unclear but based on our structure-function studies, we can propose that rather than directly influencing Cdr1 interaction with Wee1, for which there is no evidence so far, they may embed Cdr2 in a tight protein complex where Cdr2 conformation may be strongly constrained. Indeed, Cdr2 possesses a long spacer between its functional N- and C-terminal domains and may otherwise be flexible. Embedding of Cdr2 in the “node” complex may position the UBA and C-terminal domains in a way that optimizes the interactions between Wee1 and Cdr1 when Cdr2 kinase is active. Here again, structural work on the complex would be necessary to confirm this model.

Functional conservation of SAD kinases and CGN in Evolution

Cdr2 and Cdr1 are SAD kinases belonging to the AMPK family of related kinases. Members of the SAD family include the budding yeast septin kinases Gin4, Kcc4 and Hsl1, C.elegans SAD-1, and human BRSK2/SAD-A and BRSK1/SAD-B (Brain Specific Kinases).

Both cytokinetic and Wee1-dependent cell cycle regulatory functions have been reported for septin kinases in *S. cerevisiae* (Barral, Parra et al. 1999). Gin4 phosphorylates the septins which serve as a scaffold for contractile ring assembly in budding yeast and Gin4 deletion leads to abnormally shaped septa at the bud neck in combination with the deletion of the anillin like protein Bud4 (Eluere, Varlet et al. 2012). Kcc4 and Gin4, act redundantly with Hsl1 to regulate Swe1, the budding yeast ortholog of Wee1 and link entry into mitosis to proper septin organization (Barral, Parra et al. 1999).

In mammals, the expression of SAD kinases seems restricted to certain tissues. They are strongly expressed in the nervous system where they have very clear functions in neuron polarization, axon/dendrite specification and arborisation or maturation of nerve terminal (Barnes, Lilley et al. 2007; Kim, Lilley et al. 2008; Lilley, Pan et al. 2013; Lilley, Krishnaswamy et al. 2014). Although these regulations take place in post-mitotic cells, these pathways may involve a down-regulation of Wee1 activity by SAD kinases, since Wee1 knockdown rescued the polarization defects induced by the absence of SAD kinases (Muller, Lutter et al. 2010). Thus the Sad/Wee1 cell cycle regulatory pathway described in yeast may have be reused to control cell morphogenesis in post-mitotic cells in the brain. Whether DYRK kinases also function in upstream of these pathways becomes an intriguing possibility.

SAD-A has also been found in Langerhans islets of the pancreas where it works as an effector of mTOR. mTOR regulates the expression of SAD-A which in turn seems to play a role in controlling insulin secretion, actin remodeling, islet mass and most importantly islet β-cell size (Nie, Han et al. 2013; Nie, Liu et al. 2013).

This fact is reminiscent of the function of Cdr2 and Cdr1 in fission yeast where they were first described as mutants that would fail to show an adaptative response to nitrogen starvation. Wild type fission yeast cells get shorter at division when switched to mediums with poor nitrogen sources, but both *cdr1Δ* and *cdr2Δ* showed no reduction in cell size. This fact gave them their Cdr (Change Division Response) name. Strikingly, a recent report also provides a first link between Cdr2 and the TOR pathway: a TOR inhibitor that blocks its catalytic activity was shown to induce a reduction in cell size before growth arrest resulting from a Polo and Cdr2 kinase-controlled drop in Wee1 levels (Atkin, Halova et al. 2014).

These new findings raise the possibility of much larger functional conservation between the fission yeast CGN and the wealth of mammalian DYRK/Sad/Wee1/Anillin-dependent events than previously anticipated. A lot of new questions need to be addressed. Are mammalian SAD kinases inhibited by DYRK kinases as it happens in pombe? Is anillin interacting with mammalian SAD kinases and what could be the implications for neuron polarity, or insulin secretion? Are SAD kinases expressed in more proliferating tissues or during early development of multicellular organisms? Finally, Wee1 has recently emerged has an important target for cancer treatment (Stathis and Oza 2010; Do, Doroshow et al. 2013). Whether SAD kinases play a role as a modulator of Wee1 activity in cancer progression or during cancer therapy may be another important question with medical relevance to address in the future.

V.

Synthèse en Français

V. Synthèse en Français

A. INTRODUCTION

La cellule doit coordonner la division cellulaire au niveau spatial et temporel. Le choix d'où et quand se diviser est primordial afin d'assurer la viabilité cellulaire, et donc la survie de l'organisme que ce soit un organisme unicellulaire ou pluricellulaire. Dans cette thèse, l'objectif est d'approfondir la compréhension des mécanismes de coordination entre la mitose et la cytocinèse qui garantissent leur succès.

La levure *Schizosaccharomyces pombe* ou levure fissipare est grâce à sa simplicité morphologique, sa croissance stéréotypée et sa manipulation génétique aisée un très bon modèle en biologie cellulaire pour étudier les processus du cycle cellulaire tels que la mitose ou la cytocinèse. C'est pour cela que *S.pombe* est le modèle utilisé dans cette thèse.

C'est chez *S.pombe* que les premiers cribles génétiques permettant d'identifier les composants de l'horloge biochimique qui contrôle la progression du cycle cellulaire ont été faits (Nurse, Thuriaux et al. 1976; Nurse and Thuriaux 1977; Nurse and Thuriaux 1980).

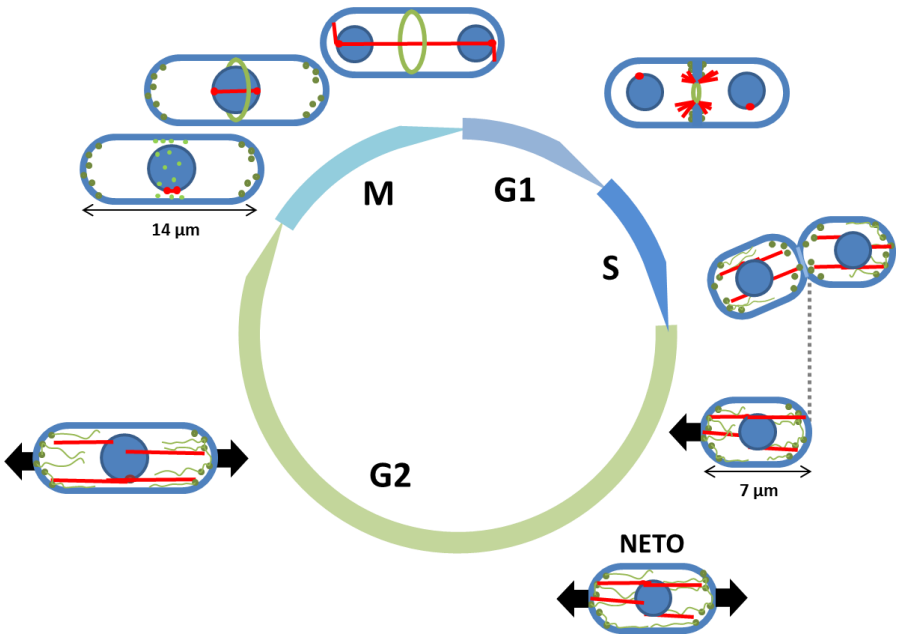

Figure F1 : le cycle cellulaire chez la levure *S.pombe*

S.pombe a une morphologie et un patron de croissance très simple. La croissance polarisée de *S.pombe* est régulée au cours de son cycle cellulaire qui dure de 2 à 3 heures. Les cellules ont une forme de bâtonnet et elles poussent d'abord de manière monopolaire par l'ancienne extrémité (celle qui existait avant la division). Puis, très tôt dans la phase G2, à environ un tiers du cycle cellulaire, un nouveau site de croissance polarisée apparaît à la nouvelle extrémité qui se met également à pousser : c'est le processus que l'on nomme NETO (*New End Take Off*). Lorsque ces cellules atteignent une longueur d'environ 14 µm, la croissance s'arrête et elles entrent en mitose. Les cellules assemblent en leur centre un anneau contractile acto-myosique. Ensuite, cet anneau se contracte de manière concomitante à la formation d'un septum, ce qui aboutit à la formation de deux cellules filles séparées physiquement tardivement en fin de mitose. L'abscission correspond à la digestion du septum par des glucanases.

La régulation de la taille cellulaire.

Dans un tissu cellulaire ou dans une culture d'organismes unicellulaire de la même espèce, les cellules ont la tendance à avoir la même taille. Ce fait suggère l'existence de mécanismes de contrôle de la taille des cellules. Le contrôle de la taille cellulaire est important pour la physiologie de la cellule puisque la taille a un impact sur plusieurs fonctions cellulaires. Pour parvenir à avoir une homéostasie cellulaire en termes de taille, les cellules ont besoin d'un équilibre entre croissance et division cellulaire. L'existence de mécanismes de contrôle de la taille cellulaire est connu depuis longtemps (Marshall, Young et al. 2012) Il a été particulièrement bien décrit chez les levures *S.pombe* et *S.cerevisiae* (Turner, Ewald et al. 2012). Recemment, quelques études ont confirmé l'existence de mécanismes de contrôle de la taille cellulaire chez les eucaryotes supérieurs (Tzur, Kafri et al. 2009; Kafri, Levy et al. 2013) indiquant que l'existence de ces mécanismes est probablement universelle.

En dépit de l'importance de la question, aucun mécanisme moléculaire n'a été proposé jusqu'à très récemment.

L'horloge biochimique du cycle cellulaire

Chez tous les eucaryotes la progression du cycle cellulaire est contrôlée par des protéines dont la concentration varie périodiquement à chaque cycle cellulaire, les cyclines. Les cyclines s'associent avec les CDKs (*Cyclin Dependent Kinase*), et chaque combinaison de cycline-kinase a une spécificité pour des substrats différents et est typique d'une phase particulière du cycle cellulaire. Chez *S.pombe* il existe une seule Cdk (Cdc2) et quatre cyclines (Cig1, Cig2, Puc1 et Cdc13) (Hagan, Hayles et al. 1988; Bueno, Richardson et al. 1991; Forsburg and Nurse 1991; Connolly and Beach 1994). La quantité de Cdc2 reste stable pendant tout le cycle cellulaire mais celle des cyclines oscille au cours du cycle. Cdc13 est la cycline mitotique qui, combinée avec Cdc2, forme le complexe Cdk1 contrôlant l'entrée en mitose.

L'activation de Cdc2 est fortement régulée pendant tout le cycle. Une légère activation permet le passage de la cellule à la phase S du cycle cellulaire et la duplication du DNA. Par contre le passage en mitose demande une forte activation de Cdk1. L'activité de Cdk1 pendant G2 est régulée négativement par la kinase Wee1, qui phosphoryle la tyrosine 15 de Cdc2 et l'inhibe, et la phosphatase Cdc25 qui enlève le phosphate et lève l'inhibition. L'équilibre entre ces deux molécules régule donc l'activation de Cdk1 et l'entrée en mitose.

Chez S.*pombe,* Wee1 est à son tour inhibée par les kinases Cdr1 et Cdr2 (Russell and Nurse 1987; Young and Fantes 1987). Cdr2 et Cdr2 phosphorylent Wee1 directement et causent son inhibition (Coleman, Tang et al. 1993; Parker, Walter et al. 1993; Wu and Russell 1993; Kanoh and Russell 1998). Cdr2 et Cdr1 se localisent pendant l'interphase dans des nœuds au cortex médian de la cellule.

Dans les nœuds médians corticaux on peut trouver 8 protéines différentes : Cdr1, Cdr2, Wee1, Mid1, Blt1, Gef2, Nod1 et Klp8. Cdr2 est le principal organisateur de ces structures puisque l'absence du reste des composants des nœuds n'a pas d'impact sur la localisation de Cdr2. En revanche, l'absence de Cdr2 affecte la localisation du reste de protéines des nœuds (Moseley, Mayeux et al. 2009).

Régulation de l'activité des nœuds par le gradient du facteur de polarité Pom1

Pom1 est une kinase de type DYRK qui forme un gradient émanant des extrémités de la cellule avec des concentrations décroissantes vers la région centrale de la cellule. Pom1 à une fonction dans l'établissement de la polarité cellulaire puisque qu'elle est nécessaire à l'activation de la croissance bipolaire,, mais cette kinase a aussi une fonction dans la régulation du cycle cellulaire et une fonction en cytocinèse. Pom1 régule négativement la distribution corticale des nœuds corticaux organisés par Cdr2 et en limite la localisation au cortex médian de la cellule. De la même façon Pom1 a aussi un effet négatif sur l'activité kinase de Cdr2 (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009; Bhatia, Hachet et al. 2014; Deng, Baldissard et al. 2014; Rincon, Bhatia et al. 2014).

<u>- Régulation du cycle cellulaire par la voie Pom1-Cdr2-Cdr1-Wee1</u>

Il y a quelques années le premier modèle biochimique proposant une régulation du cycle cellulaire par la taille des cellules a été proposé (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009). Selon ce modèle dans des cellules jeunes et courtes une concentration élevée de Pom1 dans la région médiane de la cellule inhibe Cdr2, qui à son tour ne peut pas inhiber Wee1, bloquant ainsi l'entrée en mitose. Mais losrque la cellule s'allonge par croissance polarisée, la région médiane contenant les noeuds corticaux s'éloigne de la source du gradient permettant l'activation de Cdr2 et l'entrée en mitose. Dans les années qui ont suivi la publication de ce modèle des modifications et apports ont été ajoutés au modèle. En particulier, il a été montré que Pom1 (kinase inhibitrice) antagonise l'activation de Cdr2 par

la kinase Spp1 (kinase activatrice). On a aussi découvert que la concentration de Pom1 dans la région médiane de la cellule ne diminue pas quand les cellules s'allongent comme proposé au départ, mais que la zone de concentration minime de Pom1 l'élargit (Deng, Baldissard et al. 2014). Enfin, Pom1 a un effet à deux niveaux sur Cdr2, sur son activité, qui peut être perturbée par une faible inhibition de Pom1, et sur l'assemblage des nœuds, dont l'altération requièrt un haut niveau d'inhibition de Pom1 (Bhatia, Hachet et al. 2014; Rincon, Bhatia et al. 2014).

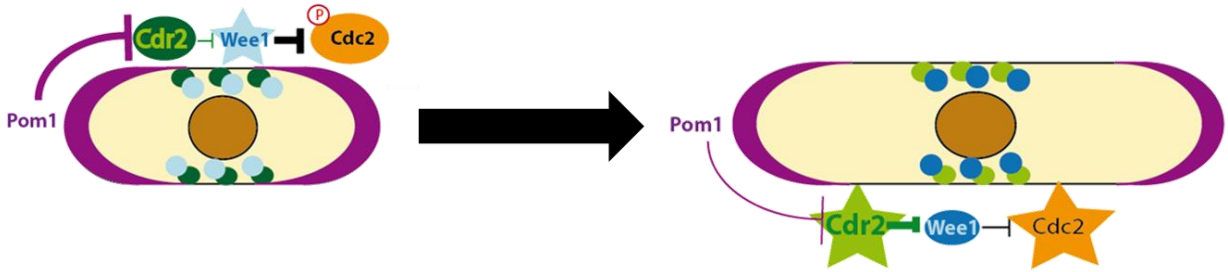

Figure F2 : Influence de Pom1 sur les nœuds et l'entrée en mitose

- Régulation de la cytocinèse par la voie Pom1-Cdr2-Mid1

Mid1 est l'homologue fonctionnel de la protéine Anillin chez les eucaryotes supérieurs. La fonction de Mid1 est de recruter les composants de l'anneau contractile nécessaire à la cytocinèse, la dernière étape du cycle cellaire permettant la séparation des deux cellules filles grâce à un anneau contractile acto-myosique. En effet, pour promouvoir l'assemblage de l'anneau contractile, Mid1 activée par Plo1 recrute séquentiellement les composants essentiels de l'anneau contractile, en particulier la myosin II, la formine Cdc12 qui nuclée l'actine et la protéine F-Bar Cdc15 qui lie la membrane plasmique (Almonacid 2011, padmanabathan, Laporte 2011). Les noeuds corticaux matures se compactent alors en anneau contractile. Ceci dépend d'évènements de traction entre les noeuds matures réalisés par des filaments d'actine nucléés par les noeuds, en interaction transitoire avec les filaments de myosin II des noeuds avoisinants.

Comme Cdr2 contrôle la localisation de Mid1 et à son tour Pom1 contrôle la localisation de Cdr2, Pom1 a un effet sur le positionnement correct de l'anneau contractile au milieu de la cellule en pré-positionnant les noeuds précurseurs de l'anneau en position médiane (Bahler and Pringle 1998; Celton-Morizur, Racine et al. 2006; Almonacid, Moseley et al. 2009).

En l'absence de Cdr2, Mid1 a un autre moyen pour assembler l'anneau là où le noyau se trouve. Une grande quantité de Mid1 localise au noyau pendant interphase. A l'entrée en mitose Mid1 est phosphorylé par la kinase Plo1 et massivement exporté en dehors du noyau et s'attache à la membrane plasmique à proximité du noyau grâce à son propre domaine d'association aux lipides, une hélice amphiphylle. Ces évènements couplent la position du plan de division à celle du noyau. et favorisent une ségrégation correcte des chromosomes. Comme le noyau est lui-même centré par les microtubules, ce second mécanisme est redondant avec la voie Pom1-Cdr2-Mid1 qui assure pour sa part une division équitable du cytoplasme (Almonacid, Moseley et al. 2009; Almonacid, Celton-Morizur et al. 2011).

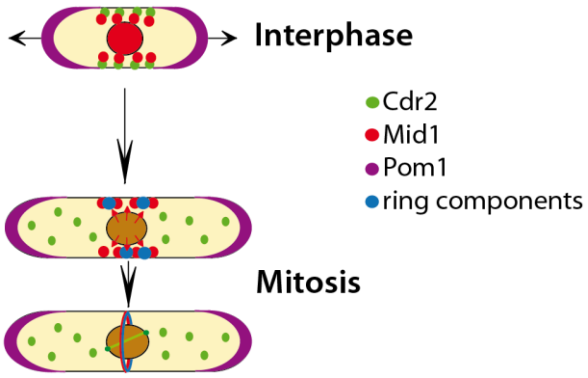

Figure F3 : Influence de Pom1 sur les nœuds et la cytocinèse. (Almonacid, Moseley et al. 2009)

B. RESULTATS ET CONCLUSIONS

Deux projets ont été développés pendant cette thèse. Le premier concerne la fonction de la nouvelle protéine des noeuds corticaux Blt1 et a mis en évidence son rôle dans l'ancrage des noeuds médians au cortex en association avec Mid1 en début de mitose, lors de l'initiation de l'assemblage de l'anneau contractile. Le deuxième concerne l'organisation des nœuds corticaux par Cdr2 et son impact sur la voie de régulation de Wee1 promouvant l'entrée en mitose.

Fonction de Blt1 dans l'ancrage des noeuds corticaux médians en début de mitose

L'ancrage au cortex de Mid1, le facteur majeur de définition du plan de division, est médiée par Cdr2 pendant interphase. Cdr2 possède un domaine d'interaction avec les lipides en C-terminus. Cependant, à l'entrée en mitose, Cdr2 quitte la région médiane alors que Mid1 est exportée du noyau et se concentre au cortex médian. Mid1 s'associe alors à la membrane plasmique à travers son propre motif d'association aux lipides composé d'une hélice amphiphylle C-terminale et d'une région polybasique ainsi que d'un domaine PH (Celton-Morizur, Bordes et al. 2004; Saha and Pollard 2012). Cependant, il a été observé que le N-terminus de Mid1 est aussi fonctionnel et capable d'assembler des anneaux dans la région centrale de la cellule alors qu'il ne possède pas de domaine de liaison menbranaire. Ce résultat a mis en évidence l'existence d'un troisième mécanisme d'ancrage à la membrane pour Mid1.

Pendant cette étude, en collaboration avec l'équipe de James Moseley, nous avons mis en évidence que ce troisième mode d'ancrage dépend de la protéine Blt1 qui intéragit indirectement avec la région comprenant les acide aminés 300-350 de Mid1.

Blt1 a été décrite récemment (Moseley, Mayeux et al. 2009) et n'avait pour lors pas de fonction connue. Blt1 a été d'abord identifiée comme une protéine que interagissait avec Cdc15, une protéine F-BAR essentielle pour l'assemblage de l'anneau contractile. La protéine

Blt1 a un patron de localisation particulier puisqu'elle se forme d'abord des nœuds corticaux aux extrémités de la cellule en tout début de cycle, puis des nœuds qui co-localisent avec Cdr2 dans la région médiane de la cellule (Moseley, Mayeux et al. 2009(Akamatsu, Berro et al. 2014). Blt1, contrairement à Cdr2, reste dans la région médiane de la cellule en début de mitose et s'associe ensuite à l'anneau contractile quand celui-ci se forme par compaction des noeuds. Ainsi, même si la localisation de Blt1 pendant interphase est dépendante de Cdr2, elle devient indépendante de Cdr2 pendant la mitose.

Une dissection moléculaire minutieuse nous a permis d'identifier le domaine d'ancrage à la membrane de Blt1 correspondant, encore une fois, à la partie C-terminal de la protéine. Elle est constituée d'un domaine riche en acides aminés basiques, qui établissent des interactions électrostatiques avec des lipides acides dans la membrane. Un mutant de délétion de ce domaine a été nommé Blt1Δ5. L'analyse de ce mutant a permis de mettre en évidence que Blt1 sert à l'ancrage membranaire des précurseurs de l'anneau contractile à l'entrée en mitose, quand Cdr2 se détache du cortex, en coopération avec Mid1. Ainsi des défauts combinés de l'ancrage de Blt1 et de Mid1 (double mutant blt1Δ5 Mid1-Nter) ont de forts défaut de position du plan de division. Ceux-ci sont compensés en partie si on maintient artificiellement Cdr2 sur le cortex pendant la mitose à l'aide d'un domaine de prenylation CAAX (mutant Cdr2-CAAX).

Le domaine de Blt1 qui établit les interactions avec le reste de composants des nœuds en interphase est lui localise dans les premiers 79 acides aminés de la protéine, et le mutant correspondant s'appelle Blt1Δ1 (figure F4).

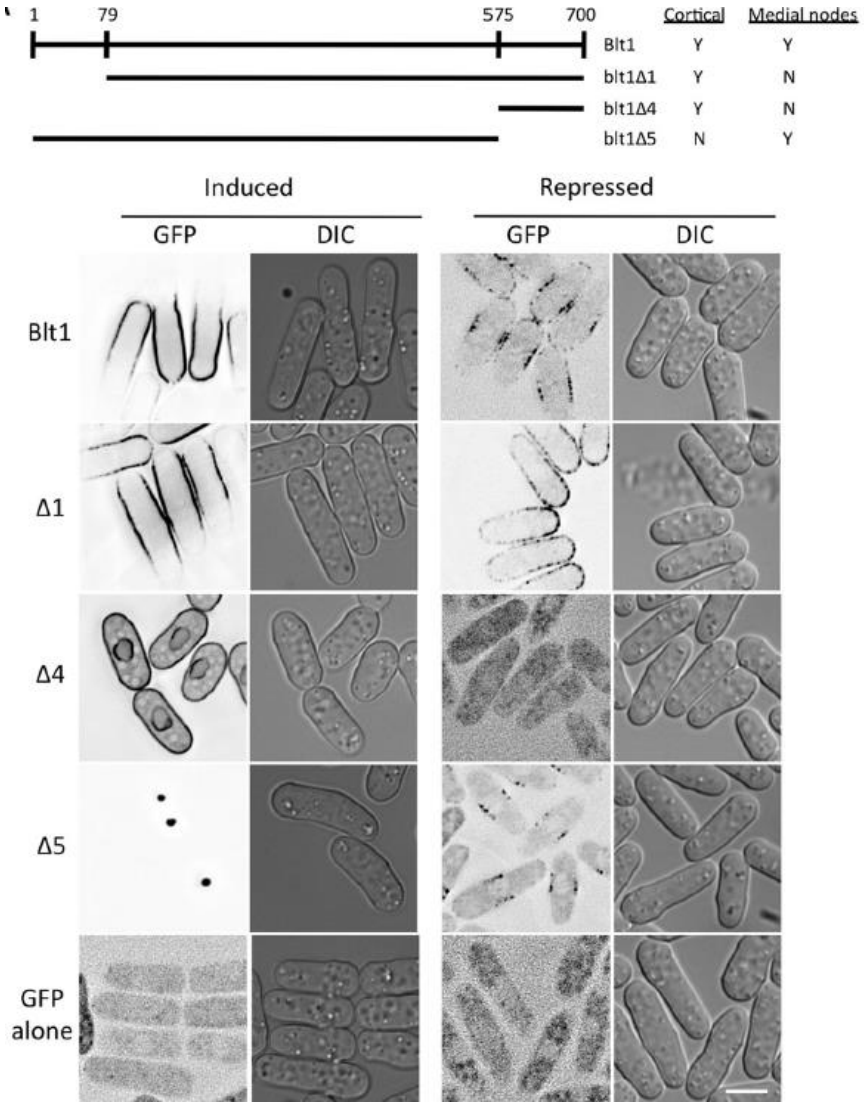

Figure F4 : Dissection moléculaire de Blt1 es ses domaines.

(Guzman-Vendrell, Baldissard et al. 2013)

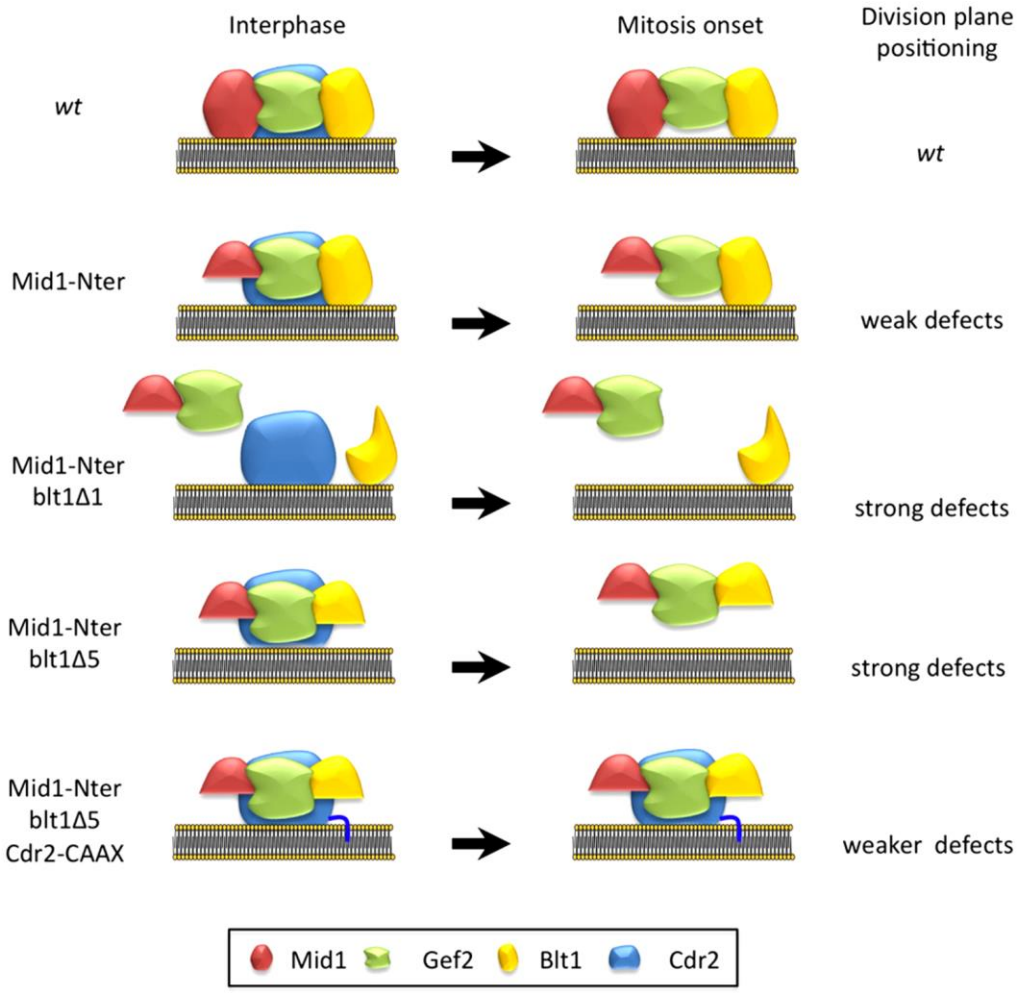

Figure F5 : Modèle fonctionnel pour l'ancrage cortical des précurseurs de l'anneau contractile à l'entrée en mitose. (Guzman-Vendrell, Baldissard et al. 2013)

Nos travaux, ainsi que d'autres études publiées en parallèle ont enfin mis en évidence que l'interaction entre Mid1 et Blt1 n'est pas directe mas elle est médiée par les protéines Gef2 et Nod1 (Ye, Lee et al. 2012; Jourdain, Brzezinska et al. 2013; Zhu, Ye et al. 2013).

Organisation des nœuds corticaux médians par la kinase Cdr2 et impact l'activité de la kinase Wee1

Les nœuds médians corticaux établis par Cdr2 pendant l'interphase ont deux fonctions : préétablir le plan de division au milieu de la cellule à travers Mid1 et servir comme plateforme d'inhibition de Wee1 par Cdr1 et Cdr2. Les nœuds ont donc une double fonction de régulation de l'entrée en mitose et de la cytocinèse. Aussi trouve-t-on dans ces structures à la fois des protéines comme Mid1 qui a une fonction dans la cytocinèse et Cdr1 et Cdr2 qui fonctionne dans le contrôle de l'entrée en mitose. D'autres protéines comme Blt1, Gef2, Nod1 et Klp8 semblent participer aux deux puisqu'en leur absence des défatus de taille des cellules et de cytocinèse ont été rapportés. Elucider comment Cdr2 organise ces protéines pour former une plateforme de signalisation et d'ancrage a été le deuxième objectif de cette thèse. Cette étude a abouti à un premier modèle d'organisation de la voie de contrôle de Wee1 par Cdr2.

Cdr2 et Cdr1 sont des kinases SAD appartenant à la famille de kinases AMPK. Cette famille de kinases a une série de caractéristiques communes. Elles ont un domaine serine/thréonine kinase en N-terminal qui est normalement régulé positivement à travers la phosphorylation du segment nommé "T-loop". De plus, les AMPKs ont un domaine UBA ou AID qui suit le domaine kinase et qui régule son activité. Les domaines UBA/AID sont parfois inhibiteurs parfois activateurs. Le domaine kinase et son UBA/AID sont suivis d'un long fragment d'acides aminée sans conformation précise et on trouve finalement dans la région C-terminale comporatant une zone basique et le domaine KA1 qui coopèrent pour l'ancrage à la membrane.

Dans ce travail nous avons mis en évidence que Cdr2 est une kinase AMPK typique qui a toutes les caractéristiques de la famille. Le domaine UBA de Cdr2 constitue un domaine activateur puisque son absence mène a une inhibition de toutes les fonctions de Cdr2, même si cette mutation n'affecte pas la localisation de la protéine. On a découvert que Mid1 et Wee1, les

deux effecteurs principaux de Cdr2, intéragissent avec le domaine UBA de Cdr2 de façon dépendante de l'activité kinase de Cdr2.

L'absence d'activité kinase affecte en affet la localisation de Mid1 et Wee1 au cortex. En revanche, l'absence de Cdr1, Mid1, Blt1 ou Wee1 n'a aucun effet sur l'activité kinase de Cdr2. La relation est donc unidirectionnelle.

Cdr1, est quant à elle une kinase APMK atypique sans domaine KA1. Elle interagit avec Cdr2 à travers son domaine KA1 de façon indépendante de l'activité kinase de Cdr2.

La délétion systématique de fragments de 50 acides aminés dans la région centrale de Cdr2 nous a enfin permis d'identifier deux régions dont la délétion provoque des défauts de cytocinèse et de cycle cellulaire. Ces régions correspondent aus aa 381-430 et 481-530 de Cdr2. Nous avons déterminé que la région 481-530 elle correspond à un site d'interaction avec Blt1.

Comment exposé plus tôt dans ce résumé, Blt1 interagit avec Mid1. Ceci constitue un deuxième mode d'interaction, indirect, entre Mid1 et Cdr2. De plus, une troisième interaction semble exister entre Mid1 et la partie C-terminal de Cdr2. La complexité et multiplicité de points d'interaction entre les deux protéines pourrait permettre de stabiliser l'interaction entre les deux protéines une fois la kinase Cdr2 active.

De manière surprenant, alors que Wee1 s'associe au N-terminus de Cdr2, Cdr1 s'associe à l'extrémité C-terminale séparée par la longue région centrale de Cdr2. La délétion de Blt1 cause un retard d'entrée en mitose indiquant que Blt1 participe au contrôle de l'activité de Wee1 par Cdr2 et Cdr1. D'après nos résultats sur les domaines impliqués dans la liaison de Wee1, Cdr1 et Blt1, notre hypothèse est que la liaison de Blt1 sur le domaine central de Cdr2 pourrait créer des contraintes conformationnelles et rapprocher les régions C-terminale liant Cdr1 et N-terminale liant Wee1 quand Cdr2 est active, rapprochant ainsi Cdr1 de son substrat

de phosphorylation Wee1 et favorisant l'inhibition de Wee1. Ceci constitue un tout premier modèle de régulation de l'activité de Wee1 par Cdr2

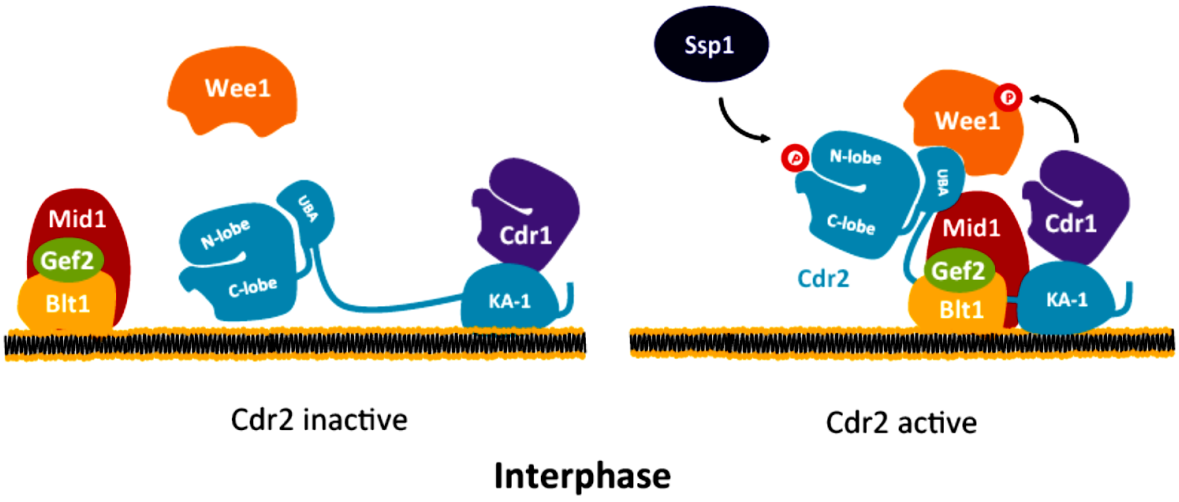

Figure F6 : Modèle sur l'architecture des noeuds et l'organisation par Cdr2 de la voie d'inhibition de Wee1

VI.
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VI. Bibliography

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Abstract

The aim of this PhD work is to bring a better understanding of the regulatory mechanism controlling cell division in space and time at the molecular level. Cell division is composed of mitosis and cytokinesis. Both processes need to be perfectly coordinated in order to guarantee genome integrity. Cell division also needs to be properly balanced with cell growth to maintain cell size constant during successive cell cycles. Temporal and spatial regulatory mechanisms ensure the coordination of these events. The fission yeast *Schizosaccharomyces pombe* is a simple rod-shaped model organism well-known for cell cycle and cytokinesis studies. In this model, we focused the work of this thesis on the medial cortical nodes, complexe protein structures that have a dual role in mitotic commitment and in division plane positioning.

Medial cortical nodes are organized by the SAD kinase Cdr2. Their localization and function is negatively regulated by the DYRK kinase Pom1 that forms a gradient emanating from the cell tips. Medial cortical nodes contain an inhibitory pathway for Wee1, promoting mitotic entry. This pathway involves the SAD kinase Cdr1, a direct inhibitor of Wee1 and has been proposed to couple mitotic entry to cell size by progressive alleviation of Pom1 inhibition when cells grow longer. Cdr2 also recruits to medial nodes the anillin Mid1 as well as a series of four additional components, Blt1, Gef2, Nod1 and Klp8, to form medial precursors for the cytokinetic contractile ring that compact into a tight ring during mitosis. Nodes medial localization, negatively controlled by Pom1 gradients, predefines thereby the division plane in the cell geometrical center.

In a first part of my thesis, I studied the previously enigmatic cortical node protein Blt1. We showed that Blt1 promotes the robust association of Mid1 with cortical nodes. Blt1 interacts with Mid1 through the RhoGEF Gef2 to stabilize nodes at the cell cortex during the early stages of contractile ring assembly. The Blt1 N terminus is required for localization and function, while the Blt1 C terminus promotes cortical localization by interacting with phospholipids. In cells lacking membrane binding by both Mid1 and Blt1, nodes detach from the cell cortex and generate aberrant cytokinetic rings. We conclude that Blt1 acts as a scaffolding protein for precursors of the cytokinetic ring and that Blt1 and Mid1 provide overlapping membrane anchors for proper division plane positioning.

In the second part of my thesis, I studied how Cdr2 scaffolds various nodes components to organize them in functional pathways promoting mitotic commitment and medial division. I showed that Cdr2 interaction with Wee1 and Mid1, depends on Cdr2 UBA domain in a kinase activity dependent manner. In contrast, Cdr1 associates with Cdr2 C-terminus composed of basic and KA-1 lipid-binding domains. Interestingly, Mid1 also interacts with Cdr2 C-terminus and may the bridge N- and C-terminal domains of Cdr2 while Blt1 associates with the central spacer region. We propose that the association of Cdr2 effectors with different Cdr2 domains may constrain Cdr1 and Wee1 spatially to promote Wee1 inhibition upon Cdr2 kinase activation.